

Interaction of SF-1 and Nur77 proteins from a gonadotrope cell
line with the promoter of the GnRH receptor gene: Implications
for gene regulation.

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The crest of the University of Stellenbosch is centered behind the text. It features a shield with various symbols, including a book and a torch, topped with a crown and flanked by two lions. The shield is surrounded by a decorative border.

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Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work (unless acknowledged otherwise) and that I have not previously in its entirety or in part submitted it at any university for a degree.

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Abstract

The regulation of gonadotropin releasing hormone (GnRH) receptor numbers in the pituitary is a crucial control point in reproduction. Pituitary sensitivity to GnRH can be directly correlated with GnRH receptor levels, which can be regulated at transcriptional and post-transcriptional level. The proximal promoter of the mouse GnRH receptor gene contains two *cis* elements bearing the consensus sequence for a Steroidogenic Factor-1 (SF-1) binding site. The distal site has previously been shown to be involved in basal and tissue-specific transcriptional regulation, whereas the function of the proximal site was not established. SF-1, a member of the nuclear receptor superfamily of transcription factors, is involved in the transcriptional regulation of a large number of genes involved in steroidogenesis and reproduction. The consensus SF-1 binding site can serve as a binding site for several members of the nuclear receptor superfamily. The aim of this study was to investigate the binding of SF-1 protein from the α T3-1 gonadotrope cell line to the two putative SF-1 binding sites in the mouse GnRH receptor promoter *in vitro*, in order to provide supporting evidence for their functional roles in GnRH receptor gene regulation. It was shown by Western blotting that SF-1 and Nur77, another nuclear receptor transcription factor, are both expressed in α T3-1 cells, in a manner that is influenced by cell culture conditions. Gel mobility shift assays using specific antibodies showed that both SF-1 and Nur77 protein in α T3-1 nuclear extracts bind to both sites in a mutually exclusive fashion. As shown by competition assays using mutated versions of the two sites, Nur77 protein had different base pair requirements than that of SF-1 protein for binding to the sites. Additionally, SF-1 mRNA was shown by Northern blotting to be increased in α T3-1 cells in response to stimulation of the Protein Kinase A (PKA) pathway by forskolin. These results highlight unexpected degeneracy in so-called "consensus" nuclear receptor binding sites. Furthermore, since Nur77 protein is involved in the stress response of the hypothalamic-pituitary-adrenal (HPA) axis, the unexpected presence of Nur77 protein in a gonadotrope cell line has potentially important implications for cross-talk between the HPA and hypothalamic-pituitary-gonadal (HPG) axes.

Opsomming

Daar bestaan 'n direkte verband tussen pituïtêre sensitiwiteit vir gonadotropien-vrystellingshormoon (GnRH) en GnRH-reseptorvlakke. Die regulering van GnRH-reseptorvlakke op transkripsionele en post-transkripsionele vlak in die pituïtêre klier is belangrik by die beheer van voortplantingsfunksies. Die proksimale promotor van die GnRH-reseptorgeen in die muis bevat twee *cis* elemente met die konsensus volgorde vir 'n Steroidogenic Factor-1 (SF-1) bindingsetel. Die distale element is betrokke by basale en weefsel-spesifieke transkripsionele regulering, maar die funksie van die proksimale element is nog nie vasgestel nie. SF-1 is 'n lid van die superfamilie van selkernreseptore en is betrokke by die transkripsionele regulering van gene verantwoordelik vir steroïedogenese en voortplanting. Die konsensus SF-1 bindingsvolgorde kan dien as bindingsetel vir verskeie selkernreseptore. Ten einde 'n beter insig ten opsigte van die regulering van die GnRH reseptorgeen te verkry, is ondersoek ingestel na die binding van SF-1-proteïen, afkomstig van die α T3-1 pituïtêre gonadotroopsellyn, aan die twee moontlike SF-1 bindingsetels in die GnRH-reseptor promotor, *in vitro*. Die Western-klad metode het getoon dat beide SF-1 en Nur77, 'n ander selkernreseptor-transkripsiefaktor, in die α T3-1 sellyn uitgedruk word. Die uitdrukking is afhanklik van selkultuurtoestande. Elektroforetiese mobiliteitsessais met spesifieke antiliggamete het getoon dat SF-1 en Nur77 proteïene in α T3-1 selkernproteïen-ekstraksies eksklusief aan beide bindingsetels bind. Nur77 proteïen benodig ander basispare as SF-1 proteïen om aan die bindingsetels te bind. Hierdie resultate dui op onverwagse degenerasie in sogenaamde "konsensus" selkernreseptor-bindingsvolgordes. Die Northern-kladmetode het ook getoon dat SF-1 mRNA vlakke in α T3-1 selle styg wanneer die proteïenkinase A (PKA) pad gestimuleer word met forskolin. Aangesien Nur77 proteïen betrokke is by die stres-respons van die hipotalamus-pituïtêre klier-adrenale (HPA) aksis, hou die onverwagse teenwoordigheid van Nur77 proteïen in 'n gonadotroop-sellyn potensieel belangrike implikasies in vir kommunikasie tussen die HPA-aksis en die hipotalamus-pituïtêre klier-gonadale (HPG) aksis.

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List of abbreviations

AF-2	activation function-2
AHC	adrenal hypoplasia congenita
ANOVA	analysis of variation
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CRE	cAMP response element
CREB	CRE-binding protein
DAX-1	<u>d</u> osage-sensitive sex reversal - <u>A</u> HC critical region on the <u>X</u> chromosome, gene <u>1</u>
DBD	DNA-binding domain
DEPC	diethyl pyrocarbonate
DMSO	dimethylsulfoxide
DTT	dithiothreitol
Egr-1	early growth response-1
ELP	embryonal long terminal repeat binding protein
ER	estrogen receptor
<i>ftz</i>	<i>fushi tarazu</i>
FTZ-F1	<i>fushi tarazu</i> factor 1
GnRH	gonadotropin releasing hormone
GnRH-RE	GnRH response element
GR	glucocorticoid receptor
GRAS	GnRH receptor activating sequence
GSE	gonadotrope-specific element
GSEB1	gonadotrope-specific element binding protein 1
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal

LB	Luria-Bertani medium
LBD	ligand-binding domain
MAPK	mitogen-activated protein kinase
MIS	Müllerian inhibiting substance
N-CoR	nuclear receptor co-repressor
NGFI-A	nerve growth factor induced gene A
NBRE	NGFI-B response element
Nurr1	Nur-related factor-1
NurRE	Nur77 response element
25-OHC	25-hydroxycholesterol
PACAP	pituitary adenylate cyclase activating polypeptide
PAP	peroxidase anti-peroxidase
PBS	phosphate-buffered saline
PGBE	pituitary glycoprotein hormone basal element
PKA	Protein Kinase A
PKC	Protein Kinase C
PMSF	phenylmethane sulfonyl fluoride
RARE	retinoic acid response element
RXR	retinoic X receptor
SF-1	Steroidogenic Factor-1
SRC-1	steroid receptor co-activator-1
StAR	steroidogenic acute regulatory protein
SURG	sequence underlying responsiveness to GnRH
TAE	Tris-acetate-EDTA
TE	Tris-EDTA
WT-1	Wilms' Tumor 1

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Vir David, wat daar was van die eerste bladsy tot die laaste

Chapter 1: Introduction

1.1: Nuclear receptors

1.1.1) The nuclear receptor superfamily

1.1.1.1) The hormone receptor family: hormone-activated transcriptional regulators

During the first half of the 20th century, hormones were identified because of their association with known human diseases, and their effects on development, differentiation and physiology. Such hormones included the sex and adrenal steroid hormones, thyroid hormones, retinoids and vitamin D3. However, the mechanism by which these hormones exerted their functions was unknown. Experiments performed in 1966 with radiolabelled hormone ligands indicated the presence of binding proteins that translocated out of the cytoplasm into the nucleus, providing the first evidence of a link between physiological events and transcriptional regulation. Ten years later, the binding of steroid hormones to high affinity receptors was shown, and in the mid 1980's the receptors for glucocorticoids (GR) (Hollenberg *et al.*, 1985) and estrogen (ER) (Green *et al.*, 1986) became the first cellular transcription factors to be cloned (reviewed in Mangelsdorf *et al.*, 1995).

The cloning of the thyroid receptor due to its homology with the GR and ER led to the realization that chemically unrelated hormones bind to structurally related receptors and, so, the idea of a hormone receptor superfamily was born. At that stage receptors in this family were characterized by the fact that they have two conserved domains, a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD). The DBD contains two highly conserved zinc finger motifs that distinguish the nuclear receptors from other DNA-binding proteins. The LBD is responsible for hormone recognition and thereby ensures selectivity and specificity of the physiological response to the hormone. The LBD can therefore be regarded as a “molecular switch” that transforms the receptor to its transcriptionally active state upon ligand binding (Mangelsdorf *et al.*, 1995).

Unlike peptide hormones and growth factors that bind to cell-surface receptors, fat-soluble steroid hormones pass through the lipid double layer of the cell membrane and bind to intracellular receptors within their target tissues. Therefore, when hormone-responsive genes were identified in these target tissues, it initiated the early characterization of a steroid hormone signaling pathway. This, in turn, led to the formulation of the classic model of steroid hormone action: binding of a hormone to its cognate receptor facilitates an allosteric change in the complex, enabling it to bind to high-affinity sites on DNA and control transcription from these sites (Mangelsdorf *et al.*, 1995).

Hormones mediate their functions through short *cis*-acting DNA sequences (± 20 bp) that are required for hormonal activation of transcription. These sequences are known as hormone response elements (HRE's). Hormone response elements function independently of position and orientation, but depend on the presence or absence of ligand. Transcriptional regulation therefore follows from the binding of hormone-receptor complexes to HRE's on DNA (Evans, 1988). HRE's are typically palindromic sequences and steroid hormone receptors bind to these as homodimers (Mangelsdorf & Evans, 1995).

1.1.1.2) An ever-expanding superfamily

By 1990, the hormone receptor superfamily encompassed 15 receptors for all the known fat-soluble hormones. These receptor proteins were purified and characterized through the use of their respective hormones and antibodies, followed by the cloning of their cDNAs. By employing these cDNAs, low stringency hybridization screening and cloning led to the discovery of a whole array of new family members without any known ligands. These receptors are commonly known as orphan receptors. The identification of the insect ecdysone receptor as a member of the nuclear receptor superfamily also indicated the universal nature of these receptors in all animals. By 1995, the superfamily had already expanded to more than 150 members from vertebrate and invertebrate animals (Mangelsdorf *et al.*, 1995).

The expansion of the nuclear receptor superfamily lead to the formulation of a more detailed model for nuclear receptor domain structure. This model was as follows: each receptor has six domains, designated A to F from N-terminus to C-terminus. The variable N-terminal end of the protein contains domain A/B, whereas the DNA binding domain is domain C. The remainder of the protein constitutes the hinge domain D, the ligand binding domain (E) and the C-terminal domain F. The first activation domain, AF-1, is contained in domain A/B, and the second activation domain, AF-2, is situated at the extreme carboxy-terminal end of the LBD. The DBD contains two zinc fingers and a carboxy terminal extension (CTE). The DBD is also referred to as region I. The LBD has many overlapping functional domains, including those for nuclear localization, dimerization and transactivation. Two areas within the LBD show extremely high levels of conservation between all nuclear receptors, and they are termed regions II and III. (Mangelsdorf and Evans, 1995, Burris *et al.*, 1996).

With regards to their dimerization behaviour and DNA-binding properties, the members of the superfamily can be divided into four classes. Class I represents the classic steroid receptors, which function as ligand-induced homodimers binding to inverted repeats of DNA half-sites. Class II includes all the remaining ligand-dependent receptors. Class III and IV contain most of the orphan receptors, with class III receptors binding as homodimers to direct repeats and class IV receptors binding single core sites as monomers. COUP-TF (chicken ovalbumin upstream promoter transcription factor) can be regarded as a class III receptor, whereas SF-1 (steroidogenic factor 1) and Nur77 can be grouped under class IV. However, the division between the classes is not always clear, since COUP-TF does not always bind as a homodimer, and both COUP-TF (Sugiyama *et al.*, 2000) and Nur77 (Perlmann and Jansson, 1995) can also interact with RXR (reviewed in Mangelsdorf *et al.*, 1995 and Mangelsdorf & Evans, 1995).

1.1.1.3) Orphan receptors

Orphan receptors have no identified ligands (reviewed in Mangelsdorf and Evans, 1995). For some of them, the expectation exists that novel ligands will be discovered, while others might be identified as having activating or repressing activities without the need for a ligand. Other orphan receptors are suspected to be modulated by post-translational modification, possibly in response to signal transduction pathways (Mangelsdorf *et al.*, 1995). Phosphorylation would therefore be a potential mechanism of modulating receptor activity. Another emerging mechanism of transcriptional control through orphan receptors is the cross-talk between different classes of nuclear receptor transcription factors. An example of this would be the inhibition of AP-1 action by the glucocorticoid, retinoid and thyroid hormone receptors, via direct or indirect interactions of AP-1 with these receptors (Pfahl, 1993). This mechanism allows genes to be under hormonal control without the hormone receptor actually binding to DNA (reviewed in Beato *et al.*, 1995).

Comparing the sequence recognition, DNA-binding, dimerization and ligand-independent activities of orphan receptors allows us to group them into four classes of their own (Mangelsdorf and Evans, 1995). Orphan receptors of the first class heterodimerize with the retinoic X receptor (RXR). They require this dimerization in order to bind to their own response elements. The heterodimers can be responsive to 9-*cis* retinoic acid (the ligand for RXR), a putative ligand for the orphan receptor, or both. Their response elements typically consist of hexad (six base pair) repeats arranged as direct or symmetrical repeats.

Class 2 orphan receptors, e.g. COUP-TF, bind DNA as homodimers and are usually strongly constitutive activators or repressors. This suggests possible ligand independence. The members of the COUP-TF family are strong dominant repressors of basal transcription and receptor transactivation. COUP-TF represses transcription by direct competitive binding and a strong carboxy-terminal repression domain. It can also form a transcriptionally inactive complex with

RXR, decreasing the availability of the latter for transcriptional activation. COUP-TF may therefore serve as a general negative regulator, keeping hormone-responsive genes switched off in the absence of hormone. Class 3 receptors, e.g. SF-1 and Nur77, are transcriptional activators that bind DNA as monomers. The consensus binding sites for these receptors all include the nuclear receptor half-site sequence 5' AGGTCA 3'. The fourth class of orphan receptors includes a few unique members of the superfamily. They either have only a LBD, such as DAX-1 (Zanaria *et al.*, 1994) or only a DBD, such as *Drosophila* Knirps (Thummel, 1995, and references therein).

1.1.2) The FTZ-F1 orphan nuclear receptor subclass

1.1.2.1) Identification and characteristics of the subclass

DmFTZ-F1 (*fushi tarazu* factor 1) was originally identified as a sequence-specific DNA-binding protein that binds to the *fushi tarazu* (*ftz*) gene in the fruit fly, *Drosophila melanogaster*. The *ftz* gene product is a homeobox segmentation factor of which the expression during the blastoderm stages of embryogenesis is essential for the correct development of *Drosophila* body segments. DmFTZ-F1 was shown to be co-expressed with *ftz* and to positively regulate the expression of this gene (Lavorigna *et al.*, 1991; Lavorigna *et al.*, 1993).

Among the members of the family of orphan receptors, it has emerged that there are certain proteins that bear extremely high similarity to FTZ-F1. These members are grouped into the FTZ-F1 subclass of orphan receptors, as part of class IV of nuclear receptors and class 3 of orphan receptors. They occur in a broad range of vertebrate and invertebrate organisms. Definitive features of the subclass members are a conserved DBD, a characteristic FTZ-F1 box and a highly specific P-box. The FTZ-F1 box is situated directly adjacent to the DBD and is required for high-affinity and sequence-specific DNA binding. It consists of 25 amino acids that are 100% identical in the greatest majority of subclass members. At the base of the first zinc finger in the DBD, there is also a short sequence of amino acids that is completely conserved in all FTZ-F1-containing receptors. These

amino acids, with the sequence Glu-Ser-Cys-Lys-Gly (ESCKG), constitute the P-box and are responsible for the specific recognition of the 6 base-pair half-site in the DNA binding site (Ellinger-Ziegelbauer *et al.*, 1994).

The consensus FTZ-F1 response element (FRE) contains the six bases 5' AGGTCA 3' of the nuclear receptor half-site, and three additional 5' bases (TCA) which confer FTZ-F1 receptor binding specificity (Ellinger-Ziegelbauer *et al.*, 1994). This is clearly distinct from the binding sites for hormone receptors, which contain two direct or palindromic repeats of the same element, separated by a specific number of nucleotides, depending on the receptor family involved.

1.1.2.2) Members of the FTZ-F1 subclass

Several invertebrates and non-mammalian and mammalian vertebrates express FTZ-F1 homologs, such as the silkworm, *Bombyx mori* (Sun *et al.*, 1994), the frog, *Xenopus laevis* (Ellinger-Ziegelbauer *et al.*, 1994), the chicken (Kudo *et al.*, 1997), mouse and rat (Galarneau *et al.*, 1996).

1.1.2.3) Identification of Steroidogenic Factor-1, a mouse FTZ-F1 homolog

Steroidogenic Factor-1 was originally cloned as a cell-selective regulatory protein that coordinates the expression of three steroidogenic enzymes: the cholesterol side-chain cleavage enzyme, the steroid 21-hydroxylase enzyme and the aldosterone synthase enzyme (Lala *et al.*, 1992). When the sequence of the putative DBD of the clone was compared with other proteins, it matched the corresponding area in mouse ELP (embryonal long terminal repeat binding protein), which is expressed in undifferentiated mouse embryonal carcinoma cells. ELP had previously been identified as a mouse homolog of FTZ-F1 (Tsukiyama *et al.*, 1992). SF-1 was confirmed to be another homolog of FTZ-F1, and it was established that ELP and SF-1 are splice variants of the same gene (Ikeda *et al.*, 1993).

1.2: Steroidogenic Factor-1 (SF-1)

1.2.1) Introduction

SF-1 is expressed in all the steroidogenic tissues, including the Leydig and fetal Sertoli cells in the testis, the placenta, the corpus luteum, the granulosa and theca cells in the ovary and in the adrenal cortex. In addition, it is also expressed in the gonadotrope cells in the anterior pituitary and the ventromedial hypothalamus (Luo *et al.*, 1994; reviewed in Parker, 1998). SF-1 therefore exerts its function at all three levels of the hypothalamic-pituitary-gonadal/adrenal (HPA/HPG) axes. It regulates a myriad of genes involved in reproduction, steroidogenesis and endocrine function. These include the genes for the glycoprotein hormone α -subunit and luteinizing hormone β -subunit, the cytochrome P450 hydroxylases, 3β -hydroxysteroid dehydrogenase and steroidogenic acute regulatory protein, Müllerian inhibiting substance and oxytocin, and the receptors for gonadotropin releasing hormone and prolactin (see Parker, 1998, and references therein). SF-1 has also been shown to be involved in the regulation of the receptors for adrenocorticotrophic hormone (ACTH) (Naville *et al.*, 1998) and Müllerian inhibiting substance (De Santa Barbara *et al.*, 1998b), and the high density lipoprotein receptor (Lopez *et al.*, 1999). It is also responsible for the proper development and organogenesis of the HPA/HPG axes (Wong *et al.*, 1996).

SF-1 as nuclear receptor binds to DNA as a monomer, and no evidence exists that SF-1 is able to form homo- or heterodimers. The protein has only twelve amino acids before the start of the first zinc finger of the DBD, and lacks the conventional A/B (and therefore the activation function-1) domains of other nuclear receptor family members. The amino acid sequence of the two zinc fingers is absolutely conserved between human, rat, mouse and bovine SF-1. This includes the amino acids of the P-box, at the end of the first zinc finger, and the D-box, in the second zinc finger. These boxes are responsible for the specific recognition of sequences and nucleotide spacing in receptor response elements (Umesono *et al.*, 1989). The FTZ-F1 box (also known as the A-box), that is

implicated in the recognition of the three nucleotides 5' to the AGGTCA half-site, is completely conserved in the SF-1 proteins of all four species mentioned previously. Mammalian SF-1 also has a conserved AF-2 activation domain (Wong *et al.*, 1996).

1.2.2) Identification and isolation of SF-1

Steroid hormones are essential for the co-ordination of physiological conditions in animals and humans. They regulate the electrolyte balance in the blood, lipid, protein and carbohydrate metabolism and sexual differentiation (Honda *et al.*, 1993). They act as transcriptional activators by forming steroid-receptor complexes and binding to hormone response elements in the promoters of target genes (Evans, 1988). These hormones are produced from cholesterol by the catalytic activities of cytochrome P450 steroidogenic enzymes that are expressed in a tissue-specific fashion to determine the tissue-specific production of steroid hormones (Morohashi *et al.*, 1992).

By 1990, it was well known that the steroid hydroxylase enzyme genes were responsive to cyclic AMP (Kagawa *et al.*, 1990; Ahlgren *et al.*, 1990; Lund *et al.*, 1990; Moore *et al.*, 1990). This was to be expected, since the trophic hormones (LH, FSH and ACTH) to which the hydroxylases are responsive, all mediate their effects in their target tissues through cAMP as second messenger (Rice *et al.*, 1990; Lynch *et al.*, 1993; Zhang and Mellon, 1996). It was also established, for at least some of the hydroxylase genes, that the response to cAMP was dependent on protein biosynthesis. This was indicated by the fact that cycloheximide, a protein biosynthesis inhibitor, prevented the increase of steroid hydroxylase mRNA after stimulation with cAMP (Kagawa *et al.*, 1990; Ahlgren *et al.*, 1990; Lund *et al.*, 1990). This implied that the gene for a specific transcription factor becomes activated upon cAMP stimulation, and that the increased levels of this transcription factor would in turn upregulate transcription of these genes. Other steroidogenic enzymes appeared to be regulated by cAMP in a manner independent of protein synthesis (Moore *et al.*, 1990). The co-ordinate regulation of steroidogenesis was therefore controversial and not well defined.

One recurring feature in the promoters of all the steroid hydroxylases was the absence of consensus cAMP response elements (CRE's) or, alternatively, the phenomenon that cAMP responsiveness was mediated through a promoter region not containing a consensus CRE. In 1992, it was discovered that all the steroidogenic P450 enzymes had at least one copy of a so-called Ad4 site, originally identified in the bovine *CYP11B1* gene, in their promoter regions. The Ad4 site positively regulated both basal expression and transcriptional stimulation by cAMP. The nuclear protein from bovine adrenocortical cells shown to bind this element in all the promoters investigated had a size of approximately 53 kDa and was designated Ad4 binding protein (Ad4BP). Nucleotide substitutions within Ad4 indicated that optimal Ad4BP binding was achieved by the sequence 5' TCA AGG TCA 3'. Ad4BP was found to be expressed in steroidogenic tissues, such as the adrenal cortex, ovarian granulosa cells and testicular Leydig cells (Morohashi *et al.*, 1992).

At the same time these observations were made, a conserved element in the promoters of the glycoprotein hormone α -subunit in several species was identified. This element was shown to confer gonadotrope-specific expression to the α -subunit gene, therefore the element was termed the gonadotrope-specific element (GSE). The protein that was found to bind there was called GSEB1 (Horn *et al.*, 1992).

Lala *et al.* were the first group to use the term Steroidogenic Factor-1 for this 53 kDa protein that interacts with similar regulatory elements in the promoters of the steroidogenic enzyme genes (Lala *et al.*, 1992). Honda *et al.* confirmed that SF-1/Ad4BP shared extensive sequence homology with ELP and FTZ-F1, and that it was therefore a novel member of the hormone receptor superfamily (Honda *et al.*, 1993). It was also shown that heterologously expressed SF-1 in non-steroidogenic tissues could upregulate transcription from Ad4 sites, but that this regulation was not influenced by stimulation with cAMP, despite the presence of putative PKA phosphorylation sites in the SF-1 amino acid sequence.

Shortly afterwards, it was discovered that SF-1 is also involved in the transcriptional regulation of the aromatase gene in the ovary. This extended the role of SF-1 beyond that of coordinate regulator of adrenal steroidogenesis to include gonadal steroidogenesis (Lynch *et al.*, 1993).

Barnhart and Mellon confirmed pituitary GSEB1 to be SF-1 (Barnhart and Mellon, 1994). Apart from the absence of glycoprotein hormone α -subunit, the pituitaries of SF-1 null mice also lack gonadotropin β -subunit and GnRH receptor expression (Ingraham *et al.*, 1994). The discovery that SF-1 regulated both steroidogenic enzymes and the gonadotropin hormones to which they respond, provided strong evidence that SF-1 plays a role in coordinated control of reproductive function (Barnhart and Mellon, 1994). The expression of SF-1 in the hypothalamus, and therefore at all three levels of the HPA/HPG axes, supported this model (Ingraham *et al.*, 1994).

1.2.3) The role of SF-1 in embryonic development

At the very beginning of gonadal differentiation, the urogenital ridge, which expresses SF-1, gives rise to the genital ridge and, ultimately, the bipotential (sex-indifferent) gonad. At the onset of sexual differentiation, SF-1 continues to be expressed in males, but not in females (Ikeda *et al.*, 1993; also reviewed in Luo *et al.*, 1994). During development of the embryonic testis, production of androgens (produced by the Leydig cells) and Müllerian inhibiting substance (MIS) (produced by the Sertoli cells) lead to the regression of the embryonic Müllerian ducts and facilitate the differentiation of seminal vesicles, epididymis and vas deferens. In the absence of these hormones, the Müllerian ducts persist and lead to the formation of female-specific tissues (Luo *et al.*, 1994, and references therein).

In order to investigate the role of SF-1 in embryonic development, targeted disruption of the SF-1 (*FTZ-F1*) gene was performed in mice. SF-1 null mice completely lack adrenal glands and gonads. These animals die a few days postnatally, due to corticosterone deficiency because of the loss of

adrenocortical function. Despite the complete absence of gonads, they have female internal genitalia (oviducts, uterus and upper vagina), regardless of their genotypic sex (Luo *et al.*, 1994). Luo and his co-workers found that gonadogenesis in SF-1 null mice actually initiated in a proper temporal and spatial fashion, only to show regression at a later stage. SF-1 null mice display development of the genital ridge early on, but the cells undergo apoptosis at exactly the stage where sexual differentiation should begin to manifest.

All of these results led to the realisation that SF-1 is initially responsible for adrenal and gonadal organogenesis, and eventually for the maintained and regulated expression of steroidogenic enzymes (Luo *et al.*, 1994).

1.2.4) Regulation of SF-1 gene expression

The fact that SF-1 knock-out mice display complete absence of gonadal and adrenal development indicates that SF-1 is involved in the regulation of genes responsible for the differentiation of these tissues (Nomura *et al.*, 1996). It is therefore absolutely essential that SF-1 expression be regulated in the correct temporal and spatial fashion during development. However, the mechanisms regulating SF-1 gene expression are currently not well defined. Nomura *et al.* identified two transcriptional elements responsible for the regulation of the SF-1 gene in Y-1 mouse adrenocortical cells. The first element to be identified is between position -82 and -77 in the rat SF-1 promoter, with a sequence identical to the consensus E-box element. These elements bind basic helix-loop-helix proteins (Nomura *et al.*, 1995). The protein binding to this site displayed sex-dependent expression in rat gonads, and has since been identified by Harris and Mellon to be USF (upstream stimulatory factor) (Harris and Mellon, 1998).

A year after they identified the E-box in the rat SF-1 promoter, Nomura and his co-workers discovered, quite surprisingly, that a functional SF-1 binding site resides in the first intron of the rat SF-1 gene (Nomura *et al.*, 1996). Inactivation of either the E-box or the SF-1 binding site significantly reduces the transcriptional activity of this regulatory region in Y-1 cells, indicating cooperative functioning of the two sites. The SF-1 binding site in the SF-1 gene forms part of an autoregulatory loop; SF-1 is therefore (at least partially) responsible for its own transcriptional control. Tissue differentiation from primordial embryonic cells takes place by means of the sequential expression of developmental genes at specific points in the differentiation process. The process is completed when the tissue expresses the final set of tissue-specific genes and acquires its specialized functions. In the developmental cascade of the adrenal cortex, the SF-1 gene will be located downstream from genes regulating its expression, and will in turn regulate the expression of the steroidogenic P450 enzyme genes. The presence of an auto-regulatory loop in the adrenal cortex implies that, once the loop becomes functional, the expression of the developmental genes upstream from SF-1 is no longer required to maintain SF-1 expression. Such an auto-regulatory loop has also been shown to exist for *Drosophila* FTZ-F1 and other genes involved in tissue differentiation. Nomura *et al.* also showed that the chromatin around the E-box and the SF-1 binding site in the SF-1 gene is accessible ("open") in steroidogenic tissues, but not in non-steroidogenic tissues. This indicates that tissue-specific gene expression not only depends on tissue-specific transcription factors, but also on tissue-specific chromatin structure (Nomura *et al.*, 1996).

SF-1 can also be regulated on several levels by cAMP and the PKA pathway; examples of this will be discussed in section 4.2.

1.2.5) Liganded and unliganded activation of SF-1 protein

When the orphan nuclear receptors were originally identified in 1990, it was believed that it would just be a matter of time before their ligands would be discovered as well. Today, even though many of them are still orphans, it is recognized that even orphan receptors depend on some form of additional factor, whether it is a conventional ligand or interaction with other proteins, to mediate non-constitutive activity.

1.2.5.1) 25-hydroxycholesterol: to be or not to be a ligand for SF-1

For a few years now, 25-hydroxycholesterol (25-OHC) has been considered to be a possible ligand for SF-1. Hydroxycholesterols, or oxysterols, are known to inhibit cholesterol biosynthesis and would therefore be physiologically relevant ligands to receptors involved in regulating steroidogenesis. In addition to this, the enzyme that converts cholesterol to oxysterols, cytochrome P450c27, is expressed in steroidogenic tissues. These compounds are therefore very likely to occur *in vivo* in these tissues. In 1997, Lala *et al.* provided evidence that SF-1 protein is activated by 25-OHC in non-steroidogenic CV-1 cells, in a oxysterol-specific and ligand-dependent fashion. This activation took place at physiological concentrations of 25-OHC and was dependent on the C-terminal AF-2 domain. It was therefore believed that a ligand had been found for SF-1 (Lala *et al.*, 1997).

However, a year later Mellon and Bair showed that 25-OHC could not increase transcriptional activation of six SF-1-dependent constructs above basal levels in MA-10 Leydig cells. This cell line expresses SF-1 endogenously. Furthermore, no increase was seen with additional SF-1 expression or stimulation with cAMP. Additionally, the natural endogenous levels of 25-OHC present in Leydig cells are at least fifty times lower than the levels required to facilitate SF-1 activation in CV-1 cells. Based on these results, they argued that 25-OHC could not be a natural ligand for SF-1 in Leydig cells. However, the possibility exists that in a different cellular environment, 25-OHC can still act as ligand for SF-1 (Mellon and Bair, 1998).

1.2.5.2) SF-1 protein is activated by different nuclear factors in different cellular contexts.

Ptx1 (also known as Pitx1), a member of the homeobox family of transcription factors, is a pan-pituitary factor that interacts with other cell-specific factors in the pituitary to direct lineage-specific gene expression. In the α T3-1 and L β T2 gonadotrope cell lines, it interacts with SF-1 and Egr-1 to facilitate GnRH-induced stimulation of the LH β promoter. Ptx1 contains two activation domains, one each in the amino- and the carboxy-terminus, of which the C-terminal domain seems to be interacting with SF-1. The enhancement of transcriptional stimulation by SF-1 is equal to that observed upon removal of the SF-1 ligand binding domain. Tremblay *et al.* therefore proposed that Ptx1 serves to unmask the activation function domains of SF-1 in a manner similar to conventional ligand binding. Ptx1 can therefore mimic the effect of a ligand on SF-1 and contribute to the apparent ligand-independent activity of SF-1 (Tremblay *et al.*, 1999).

Müllerian inhibiting substance (MIS, also known as anti-Müllerian hormone, AMH) is responsible for the regression of the Müllerian ducts during embryonic male sex differentiation. It is produced in the fetal Sertoli cells and its expression is strictly regulated. Despite the presence of a SF-1 binding site, SF-1 is unable to activate transcription from the MIS promoter in a heterologous system. This indicates that, for the regulation of the MIS gene, SF-1 requires a Sertoli cell-specific factor (Shen *et al.*, 1994). However, this factor is not the elusive SF-1 ligand, but rather another transcription factor, SOX9. Optimal MIS gene activation in Sertoli cells depends on the binding of SF-1 and SOX9 to their respective binding sites in the MIS promoter, and a direct protein-protein interaction between SF-1 and SOX9. This interaction takes place between the LBD of SF-1 and the transactivation domain of SOX9. It is also believed that SOX9 can induce a bend in DNA, bringing remote transcriptional elements and the proteins bound to them in close contact, in order to form a stable transcription complex (De Santa Barbara, 1998).

The regulation of the MIS gene also depends on the interaction between SF-1 and Wilms' Tumor 1 (WT1) protein. Missense mutations of this protein lead to male pseudohermaphroditism. Certain isoforms of WT1 can synergize with SF-1 through a direct interaction and greatly increase transcription from the MIS promoter. This interaction is disrupted by DAX-1, leading to a repression of transcription. The activity of SF-1 in male sex determination is therefore modulated by a positive (WT1) and a negative (DAX-1) factor (Nachtigal *et al.*, 1998).

Transcriptional activation by SF-1 can also be potentiated by SRC-1, a steroid receptor co-activator. Co-activators are thought to form a connection between nuclear receptors and the basal transcriptional machinery. The potentiation of SF-1 activity is mediated via the C-terminal activation function-2 (AF-2) domain of SF-1, and specifically the AF-2 activation hexamer. This hexamer is not sufficient on its own to facilitate the interaction with SRC-1 and the transcriptional activation by SF-1, but requires additional N-terminal domains of SF-1 (Crawford *et al.*, 1997).

Another set of transcriptional co-activators that is believed to perform a "bridging" function, p300/CBP (CREB binding protein), appear to mediate the regulation of the human cytochrome P450 side-chain cleavage gene by SF-1 in adrenal cells (Monté *et al.*, 1998). It is believed that p300 and CBP are general co-activators that can interact with a broad range of transcription factors, thereby integrating signals from various signal transduction pathways. It was also postulated by these authors that the negative cross-talk between certain members of the nuclear receptor superfamily could be the result of competition for a limited pool of co-activators within the cell (Monté *et al.*, 1998).

1.3: Other nuclear receptors that bind to SF-1-like sites to modulate gene regulation

1.3.1) Nur77

The nuclear receptor Nur77 forms part of the NGFI-B subfamily of receptors that also includes NOR-1 and Nurr1. This subfamily belongs to classes 1 and 3 of orphan receptors. Apart from being able to bind DNA as monomers, Nur77 and Nurr1 are able to form heterodimers with RXR (see Maruyama *et al.*, 1998, and references therein), and Nur77 can also heterodimerize with COUP-TF (Wu *et al.*, 1997). No evidence exists to indicate that Nur77 can interact with SF-1 protein. Nur77 refers to the mouse homolog of the receptor (Hazel *et al.*, 1988), whereas the rat homolog is referred to as NGFI-B, and the human homolog as NAK-1 (Nakai *et al.*, 1990; reviewed in Maruyama *et al.*, 1998). The members of this receptor subfamily are classified as immediate-early genes. Such genes are activated rapidly and transiently within minutes of stimulation by growth factors, and this activation is independent of *de novo* protein synthesis. A large number of them encode transcription factors that will regulate the expression of downstream genes, as a response to external stimulatory factors (Hirata *et al.*, 1993).

Nur77 is both an immediate-early gene product and a delayed-early gene product. It is widely expressed, but at high levels in especially the pituitary, adrenals and thymus (see Maruyama *et al.*, 1998). In the pituitary, it has specifically been shown to be expressed in the corticotrope cells (Okabe *et al.*, 1998). It is rapidly and transiently induced in fibroblasts as a result of stimulation by serum growth factors (Hazel *et al.*, 1988), but its expression in fibroblasts is also upregulated by a mechanism dependent on protein synthesis (Williams and Lau, 1993). Other stimuli that can induce the expression of Nur77 and other subfamily members are cAMP, phorbol esters, peptide hormones and neurotransmitters (reviewed in Maruyama *et al.*, 1998).

The consensus Nur77 binding site, 5' AAAAGGTCA 3', binds Nur77 monomers (Wilson *et al.*, 1991) and is known as the NGFI-B response element, NBRE. In 1997, a group led by Jacques Drouin identified a different Nur77 recognition sequence in the promoter of the pro-opiomelanocortin (POMC) gene. This element, the Nur77 response element, NurRE, consists of inverted repeats of a Nur77 binding site and binds Nur77 homodimers. Furthermore, it responds to physiological stimuli to which the NBRE is unresponsive. It therefore appears as if Nur77 could be regulated by different transcriptional mechanisms in response to different external stimuli (Philips *et al.*, 1997).

1.3.1.1) The role of Nur77 in the stress response

The primary reaction to stress is the activation of the hypothalamic-pituitary-adrenal axis and the release of corticotropin-releasing hormone (CRH) from the hypothalamic paraventricular nucleus. CRH increases both the transcription of the POMC gene (the precursor of ACTH), and the secretion of ACTH from the pituitary corticotropes, which will in turn cause an increase in the secretion of glucocorticoids from the adrenal cortex. Therefore, under stress conditions, the levels of circulating glucocorticoids are increased. This has a negative feedback effect on the HPA axis by inhibiting ACTH secretion and POMC expression, thereby inhibiting glucocorticoid production. Chronic stress requires glucocorticoid levels to be held at increased levels, to help maintain homeostasis, therefore an antagonistic mechanism to overcome the negative feedback of elevated glucocorticoid levels must exist (Okabe *et al.*, 1998, and references therein).

POMC gene expression is inhibited by glucocorticoids by means of a negative glucocorticoid response element (nGRE) in the POMC promoter. The promoter also has a Nur77-like binding site, overlapping the nGRE. Okabe *et al.* showed that, during the response of the HPA axis to chronic stress, Nur77 expression in the corticotropes is upregulated by CRH. They also showed that Nur77 can antagonize the negative feedback of glucocorticoids on POMC expression in corticotropes,

possibly through competition for binding to the overlapping DNA elements. Thereby, Nur77 can alleviate the inhibition of POMC gene transcription, ultimately restoring ACTH secretion. Nur77 can therefore partially overcome the negative feedback of glucocorticoids on the HPA axis during chronic stress (Okabe *et al.*, 1998).

1.3.1.2) DNA binding by SF-1 and Nur77

It is well established that most non-steroid nuclear receptors bind to direct or inverted repeats of half-sites as heterodimers with RXR. The half-site recognized by most nuclear receptors is known as an estrogen receptor half-site, bearing the sequence 5' AGGTCA 3'. Within the zinc fingers of nuclear receptors, two motifs can be found which determine binding specificity. The P box forms part of the first zinc finger and recognizes the half-site sequence. The D box is encompassed by the second zinc finger and can interact with the corresponding zinc finger on the protein partner bound to the other half-site of inverted repeats, to create a dimerization interface. Nuclear receptors also contain a T box, that is needed for "head to tail" interactions between two protein partners bound to direct repeats (Wilson *et al.*, 1993).

However, in the case of SF-1, the binding site consists of only one half-site, and the receptor binds this half-site as a monomer. No results that suggest that SF-1 can bind DNA as anything other than as a monomer have ever been published. Nur77 is also able to bind DNA as a monomer, although it also binds to DNA as a dimer with several different partners (Philips *et al.*, 1997, Wu *et al.*, 1997; also reviewed in Maruyama *et al.*, 1998). In the case of monomeric binding, the zinc fingers still bind to the DNA in exactly the same fashion as they would with receptor dimers, but the A box (close to the carboxy terminus) interacts with the bases directly 5' to the half-site. The A box, which is unique to each receptor, is therefore responsible for specific sequence recognition and determines monomer binding specificity (Wilson *et al.*, 1993).

Wilson *et al.* hypothesize that, in the instance of monomer binding, the T box makes intrapeptide contact, rather than interpeptide contact, with the A box. This stabilizes the binding of the protein monomer to DNA, and provides the necessary binding energy that is usually provided by the other partner of the dimer. Therefore, monomeric DNA binding of SF-1 and Nur77 represents a unique mechanism of DNA-receptor interaction that requires only one receptor half-site and specific 5' nucleotides (Wilson *et al.*, 1993).

1.3.1.3) The influence of phosphorylation status on DNA binding

The binding activity of Nur77 to the NBRE is impaired by *in vitro* phosphorylation of a serine residue within the A box, at position 354 (Hirata *et al.*, 1993). Davis and Lau showed that ACTH not only increases the expression of Nur77 in adrenocortical cells, but that it also leads to the hypophosphorylation of Nur77 protein *in vivo*, especially at serine 354. They also found that Nur77 from unstimulated adrenocortical cells does not bind to its consensus response element, whereas Nur77 from ACTH-induced cells readily binds to the response element. The phosphorylation status of Nur77 in adrenal cells is therefore under hormonal control. The hypophosphorylation of Nur77 in adrenal cells as a response to ACTH stimulation indicates that protein kinase A, which is activated when ACTH binds to its cell-surface receptor, does not phosphorylate serine 354 *in vivo*. PKA probably activates phosphatases that would ultimately lead to the dephosphorylation of Nur77 (Davis and Lau, 1994; Li and Lau, 1997).

Similarly, it was found that *in vitro* phosphorylation by PKA reduces the DNA binding activity of SF-1. SF-1 has a threonine residue in the A box that corresponds to serine 354 of Nur77. It is therefore possible that a mechanism similar to that which regulates Nur77 binding exists for SF-1, where the phosphorylation status of the protein affects the DNA binding activity (Zhang and Mellon, 1996).

1.3.2) The COUP-TF family

Members of the chicken ovalbumin upstream promoter transcription factor (COUP-TF) family are ubiquitously expressed and play an important role in organogenesis, neural development and cellular differentiation during embryonic development. They are also involved in the transcriptional regulation of several metabolic enzyme genes. COUP-TF's can homodimerize, or heterodimerize with RXR, and can therefore be classified as members of classes 1 and 2 of orphan receptors. They bind to a broad range of direct repeats of nuclear receptor half-sites (reviewed in Sugiyama *et al.*, 2000).

1.3.2.1) Gene regulation by COUP-TFs

COUP-TF family members can recognize a number of hormone response elements, thereby competing with receptors for binding to these elements. Moreover, they can form non-functional heterodimers with retinoic receptors, decreasing the availability of the latter for transcriptional activation. Both these mechanisms are examples of passive repression. COUP-TFs are also involved in active repression (recruiting co-repressors like N-CoR and SMRT) and transrepression (binding to transcriptional activators without binding to DNA itself) (Achatz *et al.*, 1997; Sugiyama *et al.*, 2000). They are therefore generally regarded as potent transcriptional repressors. However, a number of genes can also be activated by COUP-TFs (Achatz *et al.*, 1997, and references therein). They can also mediate the hormonal response of certain genes, such as the response of the phosphoenolpyruvate (PEP) carboxykinase gene to glucocorticoids. Lastly, they can also function as co-activators (Sugiyama *et al.*, 2000).

One example of a gene that is activated by COUP-TF is the immediate-early gene *NGFI-A* (also known as Egr-1) (Pipaon *et al.*, 1999). The product of this gene, which is broadly expressed throughout development and in adult life (Tremblay and Drouin, 1999), has been shown to be involved in growth regulation in certain tumor cell lines, and plays an important role in brain, organ

and vasculature development. The COUP-TF response element in the NGFI-A promoter is actually an Sp1 binding site, and it is believed that Sp1 recruits COUP-TF to the promoter in order to transactivate the NGFI-A gene in mouse urogenital mesenchymal cells. This activation is further enhanced by recruitment of the co-activators p300 and SRC-1. Since NGFI-A is an early growth response gene, COUP-TF might carry out its function in embryonic development by regulating transcription factors that mediate responses to paracrine signals (Pipaon *et al.*, 1999). Interestingly, NGFI-A has also been shown to be involved in the induction of the LH β subunit gene in pituitary gonadotrope cells, as a response to GnRH stimulation (Dorn *et al.*, 1999; Tremblay and Drouin, 1999). Evidence that COUP-TF is expressed in the α T3-1 gonadotrope cell line has been published (Yu *et al.*, 1998), and it is therefore possible that NGFI-A in gonadotropes is regulated in the same way as in urogenital mesenchymal cells.

1.3.2.2) The role of COUP-TF and Nur77 in retinoic acid resistance of cancerous cell lines

Retinoic acid (RA) can inhibit the growth of lung and breast cancer cells; however, certain cancer cell lines exhibit retinoic acid resistance. COUP-TF binds to RA response elements (RAREs) in the promoters of RA-responsive genes in RA-sensitive cell lines. The basal expression levels of these genes are thereby decreased, leading to an increase in their RA responsiveness. On the other hand, RA resistance is associated with increased Nur77 expression. Nur77 increases the basal, ligand-independent transcription from RAREs through direct protein-protein interactions with COUP-TF. This heterodimer is then unable to bind to the RARE, relieving the repression of COUP-TF on the RA-responsive genes. The relative levels of COUP-TF and Nur77 in lung cancer cell lines can be directly correlated with their RA sensitivity, with higher Nur77 expression associated with RA resistance (Wu *et al.*, 1997).

1.3.3) DAX-1

DAX-1 is another member of the nuclear receptor superfamily, expressed in the adrenals, gonads, pituitary gonadotropes and hypothalamus (Guo *et al.*, 1996; Yu *et al.*, 1998 and references therein), and is believed to regulate SF-1 function, specifically in developmental processes. Its name (dosage sensitive sex reversal - AHC critical region on the X-chromosome, gene 1) is derived from the fact that deletion of the gene causes X-linked adrenal hypoplasia congenita (AHC), and duplication thereof leads to male-to-female sex reversal. AHC is a developmental disease characterized by the absence of the adrenal cortex, and is frequently accompanied by hypogonadotropic hypogonadism (HHG) (Zanaria *et al.*, 1994).

DAX-1 is one of the few members of orphan receptor class 4, namely receptors that lack either a DBD or an LBD. It is included in the nuclear receptor superfamily on the grounds that it has a conserved C-terminal ligand binding domain (LBD, domain E). Included in this region, DAX-1 also has an AF-2 domain, possibly indicating a direct role for DAX-1 in transcriptional regulation. However, it completely lacks a conventional DNA binding domain. Instead, its amino terminal consists of four incomplete repeats of a 65 - 67 amino acid motif containing two putative zinc fingers. The cysteine residues of the zinc fingers are conserved in mouse and human DAX-1, and it is believed that these zinc fingers comprise a novel DNA binding domain (Burris *et al.*, 1996).

1.3.3.1) The influence of DAX-1 on transcriptional regulation by SF-1

Ito *et al.* showed that DAX-1 can interact directly and specifically with SF-1 via its non-conventional amino-terminus and inhibit SF-1-mediated transcriptional activation without disrupting the DNA binding efficiency of SF-1. The inhibitory function of DAX-1 appeared to reside in the carboxy-terminal domain, which is deleted or mutated in all known cases of AHC (Ito *et al.*, 1997). It was shown by Crawford *et al.* that DAX-1 recruits the nuclear co-repressor N-CoR to SF-1 to inhibit transactivation. The recruitment is facilitated by the carboxy-terminal domain, and

AHC C-terminal mutants are markedly impaired in their ability to interact with N-CoR (Crawford *et al.*, 1998). AHC is therefore the result of aberrant gene regulation during development and DAX-1 therefore exerts an inhibitory effect on transcriptional activation by SF-1 that is essential to achieve proper development of the HPA/HPG axes (Ito *et al.*, 1997).

DAX-1 can also inhibit transcription by binding to hairpin structures in promoter areas. Two promoters that contain sequences for such hairpin loops are those of the steroidogenic acute regulatory protein (StAR) gene (Lalli *et al.*, 1998) and, surprisingly enough, the DAX-1 gene (Zazopoulos *et al.*, 1997). The promoters of the human (Burris *et al.*, 1995) and mouse (Kawabe *et al.*, 1999) DAX-1 gene contain SF-1 binding sites, indicating that SF-1 regulates transcription of this gene. In the mouse promoter, the region containing the SF-1 binding site overlaps the sequence believed to form the hairpin loop. Therefore, assuming that SF-1 cannot bind to its recognition sequence when it is folded into a hairpin, conformational fluctuations between the double helix form and the hairpin loop could lead to the exchange of DAX-1 and SF-1 on the promoter. Thus, DNA conformation might be a critical factor in the regulation of the DAX-1 gene (Kawabe *et al.*, 1999). DAX-1 is therefore involved in its own regulation by at least two mechanisms: by inhibiting activation of the DAX-1 gene by SF-1 (Ito *et al.*, 1997, Kawabe *et al.*, 1999) and by binding to hairpin structures in its own promoter (Zazopoulos *et al.*, 1997).

In the hairpin area, there is also another cryptic nuclear receptor binding site, forming an imperfect direct repeat. This element is capable of binding both SF-1 (as a monomer) and COUP-TF protein (probably as a homodimer) in α T3-1 nuclear extracts. Full activation of the mouse DAX-1 promoter in Y1 and α T3-1 cells require monomeric SF-1 binding to both sites, where binding of COUP-TF homodimers inhibits this activation (Yu *et al.*, 1998).

1.3.3.2) The role of SF-1 and DAX-1 in the sex determination cascade

It is believed that SF-1 and DAX-1 form two components of a regulatory cascade directing the proper development of the HPG/HPA axes (Burris *et al.*, 1995; Habiby *et al.*, 1996; Crawford *et al.*, 1998). Vilain *et al.* proposes DAX-1 to be downstream from SF-1 in this cascade due to the fact that SF-1 is expressed in the mouse embryonic adrenal primordium 36 hours before DAX-1 can be detected. In addition, the phenotype of DAX-1 mutations is less severe than SF-1 null mutants (Vilain *et al.*, 1997). Sex determination also seems to be influenced by DAX-1. It is expressed in the indifferent embryonic gonad, but is down-regulated in the testis soon after sex differentiation is initiated, while it continues to be expressed in the ovary. In contrast to this, high levels of SF-1 expression continue in the testis, but not in the ovary. It therefore seems that SF-1 expression is necessary for male sex determination (probably to activate the MIS gene in Sertoli cells), and that DAX-1 is involved in female gonadogenesis (Swain and Lovell-Badge, 1997).

1.4: Gene regulation by SF-1, in concert with other intracellular factors and receptors

As mentioned before, SF-1 is involved in the regulation of many genes involved in reproductive function and steroidogenesis. However, the sites to which SF-1 is believed to bind also share great similarity with the binding sites of other nuclear receptors. Because of this sequence similarity, SF-1 response elements are often the site of competition between different nuclear factors (Bakke and Lund, 1995; Crawford *et al.*, 1995; Yu *et al.*, 1998; Zeitoun *et al.*, 1999). Therefore, the presence and possible transcriptional effects of other nuclear receptors in the cellular context of the investigation should always be taken into account. The binding of a different nuclear receptor to a specific promoter element, or even competition for binding between several receptors, could have a profound influence on gene expression.

It is interesting to note that every SF-1 binding site identified to date has basically the same pattern: a nuclear receptor half-site preceded by three specific base pairs. Binding sites structured like this are referred to as extended core sites, and this structure is consistent with the fact that SF-1 has never been reported to bind to DNA as anything other than as a monomer (Honda *et al.*, 1993; Wilson *et al.*, 1993). Possibly the only variation of the extended core site was identified by Zhang and Mellon when they indicated that the SF-1 binding site and cAMP-responsive region in the rat *CYP17* promoter actually required *twelve* base pairs for full function (Zhang and Mellon, 1996). This element therefore consists of an additional three base pairs, to the 5' side of the nine base pairs originally shown to constitute a consensus SF-1 binding site (Wilson *et al.*, 1993).

1.4.1) Gene regulation by SF-1, cAMP and other nuclear receptors

The mechanism by which SF-1 influences gene expression in different contexts is not always clear. A recurring theme is the involvement of cyclic AMP and the protein kinase A pathway, but in many cases, the mechanism has not yet been thoroughly investigated. However, from the instances in which the mechanism has been studied in more detail, it is clear that SF-1 exerts its influence at more than one level of gene expression. In the case of certain adrenal steroidogenic enzymes, SF-1 activity appears to be increased by post-translational modification through the action of PKA (Bakke and Lund, 1995; Zhang and Mellon, 1996). On the other hand, in the gonads, SF-1 transcription is upregulated by cAMP, and the increased SF-1 levels activate transcription of steroidogenic enzyme genes (Michael *et al.*, 1995; Zeitoun *et al.*, 1999). It is also possible that, in some cases, transcriptional regulation by SF-1 results from a combination of these mechanisms.

1.4.1.1) Activation of the P450c17 gene by post-translational activation of SF-1

Cytochrome P450c17 has two distinct enzymatic activities: a 17 α -hydroxylase and a c17,20 lyase activity. Together, they facilitate the synthesis of cortisol in the adrenals and androgens in the gonads. Transcription of the *CYP17* gene is regulated by ACTH in the adrenals, and by LH in the gonads, employing cAMP as a second messenger (Bakke and Lund, 1995; Zhang and Mellon, 1996). The rat *CYP17* gene promoter has no consensus cAMP response element, but contains a single SF-1 binding site, consisting of twelve base pairs. This element mediates basal transcription and the cAMP response in Y-1 mouse adrenocortical cells and MA-10 mouse testicular Leydig cells. It is believed that, in this case, the cAMP response is facilitated by the PKA-dependent post-translational modification of SF-1, since SF-1 mRNA levels do not increase in either cell line upon cAMP stimulation. CREB protein was shown not to bind to this response element (Zhang and Mellon, 1996), indicating that SF-1 alone mediates the response.

The bovine *CYP17* promoter has two elements that are responsive to cAMP. One of these elements consists of a direct repeat of the nuclear receptor half-site, separated by six nucleotides. In Y1 mouse adrenocortical cells, SF-1 and COUP-TF monomers bind to this element in a mutually exclusive manner. Mutational analysis indicated that the two proteins bind to non-identical but overlapping sequences in the response element, and probably prevent binding of the other factor by steric hindrance. Although COUP-TF is able to homodimerize, and although this element is a direct repeat, COUP-TF appears to bind there as a monomer. Stimulation of the PKA pathway by forskolin (a synthetic activator of adenylate cyclase) activates transcription of the bovine *CYP17* promoter in Y1 cells. Overexpression of SF-1 protein has a similar effect, and overexpression of SF-1 in the presence of forskolin has an even greater effect. COUP-TF, on the other hand, is unable to activate transcription via this response element, and it also represses SF-1-stimulated transcriptional activation (Bakke and Lund, 1995).

1.4.1.2) Activation of the P450 aromatase gene by increased expression of SF-1 in the ovary

Granulosa cells of the pre-ovulatory follicle increase their estrogen levels in response to FSH stimulation, an effect mediated via the PKA pathway. In bovine ovarian granulosa and luteal cells, increased expression of cytochrome P450 aromatase (the steroidogenic enzyme responsible for estrogen production) in response to cAMP requires a nuclear receptor half-site in the proximal aromatase promoter (Michael *et al.*, 1995). Several nuclear receptors can bind as monomers to this element, but it was established that it serves as a SF-1 binding site in luteal cells. It was also shown that upregulation of aromatase transcription is actually the direct result of increased SF-1 mRNA and protein levels. The cAMP response in luteal cells is therefore not caused by the modification or activation of SF-1 protein already present in the cell, but by increased SF-1 protein expression and subsequent binding. However, the two mechanisms are not mutually exclusive, and it remains possible that, along with increased SF-1 levels (Michael *et al.*, 1995), SF-1 protein could also be activated by the PKA pathway (Hosokawa *et al.*, 1998).

Endometriosis, a condition that is characterized by the presence of endometrium-like tissues outside the uterine cavity, arises from abnormally high estrogen levels in these tissues. This is due to the aberrant expression of aromatase, which is normally absent in eutopic endometrial tissues. COUP-TF binds to a single nuclear receptor half-site in the aromatase promoter and represses aromatase expression. This interaction is not very strong, since COUP-TF apparently homodimerizes in these cells and would preferentially bind to repeats of receptor half-sites. When SF-1 is present, as is the case in endometriotic tissue, SF-1 competes with COUP-TF for binding to the receptor half-site and is proposed to bind there with much higher affinity, thereby activating transcription of the aromatase gene. This activation is further enhanced by cAMP. COUP-TF therefore prevents aberrant expression of aromatase in endometrial tissue, but its inhibitory effect is competed away by increased expression of SF-1 in endometriotic tissues. However, the cause of the initial increase in SF-1 expression in endometriotic tissue is still unknown (Zeitoun *et al.*, 1999).

1.4.1.3) Regulation of adrenal P450 21-hydroxylase by SF-1 and Nur77

It is believed that SF-1, which appears to be constitutively expressed in adrenocortical cells, regulates basal levels of cytochrome P450 21-hydroxylase transcription (Crawford *et al.*, 1995, and references therein). Nur77 protein can also bind to the SF-1 binding site in the promoter of the mouse 21-hydroxylase gene. Treatment of adrenocortical cells with ACTH rapidly increases the levels of Nur77, through the action of cAMP (Crawford *et al.*, 1995, and references therein), whereas SF-1 mRNA levels remain unchanged. It also causes significant stimulation of the 21-hydroxylase gene after a few hours, via a mechanism that is dependent on protein synthesis. ACTH therefore induces an early-response gene, Nur77, that will in turn lead to increased 21-hydroxylase expression (Wilson *et al.*, 1993b, and references therein). The induction of Nur77 therefore leads to increased adrenal steroidogenesis (Crawford *et al.*, 1995).

Intriguingly, Nur77 null mice do not present with any impairment of development or reproductive function. Nur77 is not absolutely required for the maintenance of 21-hydroxylase expression, nor is it absolutely required for the restoration of steroidogenesis after glucocorticoid suppression. Thus, the HPA and HPG axes are functional even in the absence of Nur77. This suggests that other nuclear factors, such as SF-1 and Nurr1 (Nur77 related factor-1) could possibly compensate for the loss of Nur77 function (Crawford *et al.*, 1995), or that the transcriptional effect of Nur77 is only required under certain conditions.

1.4.2) Gene regulation by SF-1 in the gonadotropes

The four glycoprotein hormones consist of an α -subunit that is common to all four hormones, and a non-covalently associated β -subunit that confers hormonal specificity. In the anterior pituitary of all mammals, thyroid stimulating hormone (TSH) is produced in the thyrotropes, whereas the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), are produced in the gonadotropes. Chorionic gonadotropin is expressed in the placental trophoblasts of primates and horses (Pierce and Parsons, 1981).

1.4.2.1) Regulation of the glycoprotein hormone α -subunit

Horn *et al.* identified a gonadotrope-specific regulatory element (GSE) in the promoter of the α -subunit gene of several species. They also identified a 54 kDa nuclear protein that binds to the GSE, and named it GSEB1 (GSE binding protein 1). The protein was found to be expressed in α T3-1 gonadotrope cells, but not in any other pituitary cell lines or in placental cell lines (Horn *et al.*, 1992). GSEB1 was later identified as SF-1, based on antibody assays using a specific anti-SF-1 antibody. The GSE was shown to be necessary for tissue-specific activity, since it enhanced transcription in cell lines expressing endogenous SF-1, but not in a cell line which does not express SF-1 (Barnhart and Mellon, 1994).

Cyclic AMP plays a role in transcriptional regulation of the α -subunit gene in both the pituitary and placenta. Expression in the placenta requires a tandem repeat cAMP response element (CRE), as is the case in the primate and horse promoters, but the corresponding area in the mouse and bovine promoters consists of a single CRE sequence and its function in the placenta is greatly reduced. This provides a molecular basis for species-specific placental α -subunit expression (Bokar *et al.*, 1989). In the pituitary of all four of these species, PACAP (pituitary adenylate cyclase activating polypeptide) enhances basal and GnRH-stimulated gonadotropin production (Burrin *et al.*, 1998, and references therein) by increasing α -subunit transcription via the CRE (Burrin *et al.*, 1998).

Pulsatile GnRH administration was found to stimulate transcription from the α -subunit promoter in α T3-1 cells. Continuous GnRH stimulation was found to result in the down-regulation of the PKC pathway and the desensitization of the promoter response (Kay *et al.*, 1994). In contrast to this, it was found that continuous GnRH stimulation lead to an accumulation of endogenous α -subunit mRNA in α T3-1 cells, with no apparent desensitization. Chedrese *et al.* showed that stimulation with GnRH transiently increased activity of the α -subunit promoter, after which it declined. During this second phase of expression, the half-life of α -subunit mRNA markedly increased, leading to an accumulation of α -subunit transcripts (Chedrese *et al.*, 1994).

The mouse α -subunit promoter appears to contain two separate regions involved in conferring GnRH responsiveness. When they were investigated in isolation, the one element was directly responsive to GnRH stimulation, while the other element enhanced basal transcription. These promoter elements were named the GnRH response element (GnRH-RE) and the pituitary glycoprotein hormone basal element (PGBE), respectively, since the latter was found to be functional in thyrotropes as well as gonadotropes (Schoderbek *et al.*, 1993). A DNA-binding protein from the Ets family, known to be involved in transcriptional responses to the MAP kinase pathway, was shown to bind to the GnRH-RE, and stimulation of this pathway was found to be sufficient to

activate α -subunit transcription in α T3-1 cells. Treatment of α T3-1 cells with GnRH increased activity of the MAP kinase pathway, whereas inhibition of this pathway led to an impaired GnRH response (Roberson *et al.*, 1995). The PGBE is recognized by LH-2, a member of the LIM homeodomain transcription factor family. In the pituitary, this factor is expressed exclusively in the thyrotropes and gonadotropes, and it is not expressed in the placenta. Binding of LH-2 to the PGBE enhances transcription from the α -subunit promoter in α T3-1 cells (Roberson *et al.*, 1994), supporting the hypothesis of Schoderbek *et al.* that the PGBE confers pituitary-specific expression (Schoderbek *et al.*, 1993). Even though the GnRH-RE and the PGBE share no sequence similarity and have different individual functions, Schoderbek *et al.* propose that they cooperate to form a composite GnRH response unit (Schoderbek *et al.*, 1993).

1.4.2.2) Regulation of the LH β gene

SF-1 is involved in the transcriptional activation of the LH β subunit promoter in α T3-1 cells via a gonadotrope-specific element (GSE) similar to the one in the α -subunit gene (Halvorson *et al.*, 1996; Keri and Nilson, 1996). In α T3-1 cells, SF-1 expression levels are not influenced by GnRH, but the expression of Egr-1, an immediate early response transcription factor, is increased. This increase is mediated via the PKC pathway, which is activated upon binding of GnRH to the GnRH receptor. The higher Egr-1 protein levels enhance the interaction between Egr-1, SF-1 and Ptx-1 (a pan-pituitary factor) to stimulate LH β promoter activity (Tremblay and Drouin, 1999). In CV-1 monkey kidney cells, transcriptional activation of the LH β promoter by these three factors is dependent on the SF-1 and Egr-1 binding sites (Tremblay and Drouin, 1999). The synergy between Egr-1 and SF-1 appears to be disrupted by DAX-1, decreasing the transcriptional stimulation (Dorn *et al.*, 1999). The influence of GnRH stimulation on SF-1 expression in the pituitary remains controversial, since pulsatile GnRH administration seems to increase SF-1 expression in adult pituitaries (Haisenleder *et al.*, 1996), but not in the pituitary gonadotrope cell line L β T2 (Dorn *et al.*, 1999).

1.5: The regulation of GnRH receptor expression in pituitary gonadotropes

1.5.1) The anterior pituitary and the α T3-1 cell line

The anterior pituitary consists of five subsets of endocrine cell-types, each producing different hormones. The corticotropes, which express pro-opiomelanocortin (POMC), differentiate first during pituitary development. Other cell-types that arise later in development are the thyrotropes, which produce thyroid-stimulating hormone (TSH), gonadotropes, which produce the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), somatotropes, which express growth hormone (GH) and lactotropes, which produce prolactin (Horn *et al.*, 1992).

The α T3-1 clonal cell line represents precursor gonadotrope cells from the anterior pituitary. The cell line was created by means of targeted tumourigenesis in transgenic mice. α T3-1 cells have maintained some of the differential functions of pituitary gonadotropes, such as glycoprotein hormone α -subunit expression, synthesis and secretion, and GnRH responsiveness. They also express functional GnRH receptors, as indicated by the fact that the GnRH response in these cells is inhibited by a specific GnRH antagonist. However, the cell line does not express the glycoprotein hormone (LH and FSH) β -subunits (Windle *et al.*, 1990).

1.5.2) Gonadotropin-releasing hormone and its receptor

Gonadotropin-releasing hormone (GnRH) is the peptide hormone primarily responsible for the regulation of mammalian reproductive function. It is produced in the hypothalamic GnRH neurons, from where it is released into the hypothalamic portal system (reviewed in Kalra, 1993). The main physiological function of GnRH is to control the synthesis and secretion of the gonadotropin hormones LH and FSH from the anterior pituitary. This function is mediated by the activation of GnRH receptors on the cell surface of pituitary gonadotropes (reviewed in Clayton and Catt, 1981). The GnRH receptor is a G-protein coupled receptor and consists of seven transmembrane domains.

Its amino acid sequence predicts potential sites for N-linked glycosylation and phosphorylation by PKA and PKC (Clayton, 1989). It differs from other seven membrane domain G-protein coupled receptors in that it has no cytoplasmic tail (Naor, 1990). Hence, the functions of coupling to G-proteins, receptor desensitization and internalization are all performed by other regions in the protein (reviewed by Stojilkovic and Catt, 1995). GnRH receptors are linked to phospholipase C, which will produce inositol triphosphate (IP₃) and diacylglycerol (DAG) upon binding of the ligand, GnRH. In turn, IP₃ will lead to an increase in intracellular calcium levels, while DAG will activate protein kinase C (PKC) (see Stojilkovic and Catt, 1995, and references therein).

In the anterior pituitary, GnRH receptors are only expressed in the gonadotropes (Hyde *et al.*, 1982). Furthermore, GnRH receptors can be found in various regions of the brain, in the Leydig cells of the testis, and in the granulosa and luteal cells of the ovary. The numbers of pituitary GnRH receptors change during the estrous cycle, pregnancy and lactation (see Stojilkovic and Catt, 1995, and references therein). Castration, which increases GnRH secretion, also leads to an increase in receptor levels (Kaiser *et al.*, 1993). Most importantly, though, continuous stimulation by GnRH leads to down-regulation of the GnRH receptor, whereas pulsatile administration of GnRH increases receptor mRNA levels several fold (Stojilkovic and Catt, 1995).

It is clear that pituitary sensitivity to GnRH has a profound influence on reproductive function and can be directly correlated with GnRH receptor numbers on the cell surface. It is known that receptor numbers are regulated at post-translational level, by internalization, recycling and degradation. Receptor numbers could also be regulated at transcriptional level by the modulation of gene expression. Therefore, any factors influencing the levels of transcription of the GnRH receptor gene will have an influence on the entire reproductive system (Kaiser *et al.*, 1993).

1.5.3) Transcriptional regulation of the mouse GnRH receptor gene

1.5.3.1) The basic structure of the mouse GnRH receptor promoter

Albarracin *et al.* were the first to isolate a significant portion of the 5' flanking region of the mouse GnRH receptor gene. The original clone contained 1164 base pairs of 5' flanking region, 62 base pairs of 5' untranslated region and 460 base pairs of the protein coding sequences of exon 1. Of these, residues -1164 to +50 were cloned into a luciferase expression vector. They identified the major transcription start site to be at an adenine residue at -62, relative to the translation start site. No consensus TATA box resides near the transcription start site; instead, a TATA-like element was found approximately 30 base pairs from the start site that could function as a TATA box. By sequence analysis, Albarracin *et al.* also identified an AP-1 binding site at position -274 (relative to the transcription start site) and a consensus gonadotrope-specific element (GSE) in the 5' untranslated region, at position +48, relative to the transcription start site. The promoter reporter construct was preferentially expressed in α T3-1 cells, and had higher transcriptional activity in this cell line than in non-gonadotrope cell lines. It was also responsive to GnRH, and when it was co-transfected with GnRH receptor cDNA into GH₃ somatotrope cells, expression of the reporter construct increased upon stimulation with GnRH. The regulatory elements required for tissue-specific expression and regulation by GnRH are therefore contained within the proximal 1200 base pairs of the mouse GnRH receptor promoter (Albarracin *et al.*, 1994).

Clay *et al.* further delineated some regulatory regions within the promoter. Apart from a second, more distal transcription start site, their results also indicated the possible presence of an enhancer element between -1200 and -600¹. More specifically, they raise the possibility of a repressor element located between -600 and -500, and a basal transcriptional enhancer located between -500 and -400 (Clay *et al.*, 1995).

¹ Note that from this point onwards, all numbers are relative to the translation start site, unless stated otherwise.

1.5.3.2) Tissue-specific expression of the GnRH receptor

Clay *et al.* found that the gonadotrope-specific element (GSE) was not involved in gonadotrope-specific expression. Instead, they showed that expression in α T3-1 cells was greatly reduced upon deletion of a broad region between -500 and -200. They also found that at least two different α T3-1 nuclear proteins specifically recognized DNA sequences between -500 and -400 (whereas the same binding activity was not observed with nuclear extracts from other cell lines), and that the deletion of these 100 base-pairs severely attenuated promoter activity (Clay *et al.*, 1995). However, this region alone was not sufficient to drive gonadotrope-specific transcription from a minimal promoter. It was concluded that the region between -500 and -200 contains additional elements required for basal promoter activity. This region contains the AP-1 binding site, and a GSE homologue, located at position -245 (relative to the translation start site), capable of binding SF-1 protein. It was also found to contain an element, at position -393, that contributed to basal transcriptional activity. The promoter region containing all three of these elements was able to independently drive gonadotrope-specific expression. It was therefore concluded that gonadotrope-specific expression is mediated by a complex basal enhancer, located between -500 and -200 (Duval, Nelson and Clay, 1997).

Later that year, the same authors confirmed that a tripartite (consisting of three distinct elements) basal enhancer regulates transcription of the mouse GnRH receptor gene in α T3-1 cells. The three elements included the distal GSE homologue/SF-1 binding site previously identified, the AP-1 binding site and the element with unknown binding specificity described in their previous paper. This element was named GRAS (GnRH receptor activating sequence). Mutation of any one of the three elements caused a loss of 60% in transcriptional activity, while mutation of any two resulted in an 80% loss of activity. Simultaneous mutation of all three elements completely abolished transcription from the GnRH receptor promoter. A construct containing three copies of the GRAS

element was also able to confer α T3-1 cell-specific activity onto a heterologous promoter (Duval, Nelson and Clay, 1997b).

It was later shown that the GRAS region can be activated by activin, and inhibited by follistatin, an activin-binding protein. Activin and inhibin are established regulators of FSH expression, but their subunit mRNA levels do not differ during the estrous cycle. On the other hand, follistatin levels increase during the ovulatory LH surge, an effect that has been attributed to the upregulation of follistatin gene expression by GnRH. Hence, a feedback loop exists in which the stimulation of GnRH receptor gene expression by activin can be modulated by follistatin. In turn, GnRH can regulate follistatin levels. Hence, the relative levels and activities of activin, inhibin and follistatin could play an important role, not only in basal and gonadotrope-specific GnRH receptor expression, but also in the regulation of pituitary sensitivity to hormonal stimulation (Duval, Ellsworth and Clay, 1999).

It seems unlikely that either SF-1, which is also expressed outside the pituitary, or AP-1, of which the subunit members are ubiquitously expressed, could be solely responsible for gonadotrope-specific expression. Therefore, the GRAS element is a likely candidate to be primarily responsible for conferring cell-specific expression to the mouse GnRH receptor promoter (Duval, Nelson and Clay, 1997b).

1.5.3.3) The regulation of GnRH receptor expression by GnRH in GGH₃ cells

The GGH₃ cell line was created by stably transfecting GH₃ somatolactotrope cells with the GnRH receptor cDNA and is regarded as a useful model system to study the response of the GnRH receptor promoter to GnRH. In 1998, Lin and Conn provided evidence that cAMP is involved in the activation of GnRH receptor gene transcription in GGH₃ cells. They found that cAMP activated transcription of the GnRH receptor gene, and that it enhanced the activation of GnRH receptor

transcription by GnRH. Thus, in GGH₃ cells, GnRH can in part activate transcription from the GnRH receptor promoter via the PKA signal transduction pathway. The experiments from which these results were obtained were performed with a construct containing 1226 base pairs of promoter region directly 5' to the translation start site (Lin and Conn, 1998). It therefore contains both the GSE in the 5' untranslated region (Horn *et al.*, 1992) and the GSE homolog further upstream (Duval, Nelson and Clay, 1997).

The same authors later published new evidence that several other signal transduction pathways are involved in the response of the GnRH receptor promoter to GnRH in GGH₃ cells. They found that phorbol 12-myristate 13-acetate (PMA), an activator of the PKC pathway, stimulated GnRH receptor activity, but it did not potentiate the activation of the promoter by a GnRH agonist. However, a PKC inhibitor inhibited the response to GnRH. In addition, the response of the promoter to GnRH was shown to be dependent on the influx of external calcium ions. The MAP kinase pathway was shown to have an inhibitory effect on the promoter construct, under both basal and GnRH-stimulated conditions. Together, these results implicate several signal transduction pathways to play a role in GnRH receptor regulation in GGH₃ cells (Lin and Conn, 1999).

Conn, together with Maya-Núñez, found that a repressor element resides at position -343. According to the authors, deletion of this element caused an increase in basal transcription and GnRH-, PKA- and PKC-stimulated transcription in GGH₃ cells. They also found a cAMP-responsive region at position -107. Mutation of this element reduced the response of the GnRH receptor promoter to GnRH, and completely abolished the stimulation of promoter activity by the PKA pathway (Maya-Núñez and Conn, 1999).

1.5.3.4) The regulation of GnRH receptor expression by GnRH in α T3-1 cells

Numerous reports indicate that GnRH up-regulates GnRH receptor expression in the short term, but that expression is down-regulated in the long term (Norwitz *et al.*, 1999, and references therein). The response of a GnRH receptor promoter construct to a GnRH agonist was shown to be cell-specific. Norwitz and his co-workers identified two elements in the GnRH receptor promoter that can independently confer GnRH responsiveness onto a heterologous promoter in α T3-1 cells. One of these elements, named SURG-2 (Sequences Underlying Responsiveness to Gnrh-2), which also includes the AP-1 binding site within its boundaries, proved to be critical for the full response. The remaining element, SURG-1, was required for an optimal GnRH response. It was also shown that members of the Jun and Fos protein families recognized SURG-2, and that the AP-1 complex therefore plays an important role in conferring GnRH responsiveness. The promoter response to GnRH mediated by both SURG-1 and SURG-2 was dependent on the PKC pathway, but not the PKA pathway. However, only the promoter region that contains these two elements (-308 to -264, relative to the transcription start site) was tested for sensitivity to PKA and PKC stimulation. The full-length promoter construct (-1164 to +62, relative to the transcription start site) was not tested (Norwitz *et al.*, 1999).

The activation of the GnRH receptor promoter by GnRH in α T3-1 cells was confirmed by Brett White and his co-workers to be dependent on the AP-1 binding site, and mediated via the PKC pathway. Involvement of the MAP kinase pathway was also shown, possibly by being activated by PKC. The activation was not dependent on stimulation of the PKA pathway and it was not affected by intracellular calcium levels. The SF-1 site in the tripartite basal enhancer (Duval, Nelson and Clay, 1997b) did not appear to play any role in mediating or modulating GnRH responsiveness (White *et al.*, 1999).

1.6: The historical context of this project

1.6.1) Background from the literature

Two GSE (gonadotrope-specific element) homologues have been identified in the proximal mouse GnRH receptor promoter (Albarracin *et al.*, 1994; Clay *et al.*, 1995). The proximal element is located in the 5' untranslated region, at position -15 (site 1, for the purposes of this thesis), and the distal element at position -245, (site 2, for the purposes of this thesis). These sites share sequence similarities with the GSE originally identified in the glycoprotein hormone α -subunit promoter (Horn *et al.*, 1992). However, it was found that the proximal GSE homologue played no role in directing cell-specific expression in the α T3-1 gonadotrope cell line (Clay *et al.*, 1995). In fact, some authors argued that the proximal GSE does not have any function at all (Ngan *et al.*, 1999). On the other hand, the distal GSE homologue was shown to form part of a complex basal enhancer element, and to be involved in conferring gonadotrope-specific expression (Duval, Nelson and Clay, 1997b). However, this site does not appear to play a role in mediating the response of the GnRH receptor promoter to GnRH (White *et al.*, 1999; Norwitz *et al.*, 1999). SF-1 protein from α T3-1 cells was shown by a gel mobility shift assay to bind to site 2 (Duval, Nelson and Clay, 1997). However, for reasons that will be discussed in the following section, very few investigators had focused their efforts on site 1; hence, very little was known about the function of this element.

The GnRH response in α T3-1 cells has been found to involve the activation of the MAP kinase and PKC pathways (White *et al.*, 1999). The response is mediated via the AP-1 site that forms part of the complex basal enhancer (Duval, Nelson and Clay, 1997b; White *et al.*, 1999), and appears to be independent of the PKA pathway (Norwitz *et al.*, 1999). In contrast to this, experiments in GGH₃ somatolactotrope cells have shown that the GnRH response is mediated via the PKA pathway (Lin and Conn, 1998), and that other signalling pathways are also involved (Lin and Conn, 1999). An

atypical cAMP response element (CRE) has also been identified in the proximal promoter (Maya-Núñez and Conn, 1999).

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-410  TTTCATTTTG  TATCTGTCTA GTCACAACAG  TTTT TAGAAA  ACCTATTCAT
-360  TAAGGCTAAT  TGGATGATAT  TATAGAGTCAC  TTTCGACATC  AGAATTAGAC
-310  TCCAAGTGTC  CTTCTCACC  TACGATAAAA  AAGACGGGGC  ATCTGCTGAG
-260  GGGCTACGGT  TACACTTGGC CTTCAGGAGG  GCTTTGGCAT  GTTCTGTTAG
-210  CACTCTTTTA  GATTATAAAG  GCCGAAAAAC  AAGTTTACCT  TGATCTTTCA
-160  CGCCAAGTCC  AGAGTATCTT  GGGAAAAATA  AATTAGGCAG  AAATGCTAAC
-110  CTGTGACGTT  TCCATCTAAA  GGAGGCAGAC  ATCAACAGCT  GCGCGTTCAG
-60   TTATGATAAA  ACATCAGAAG  TAACAGGGAC  TCCACTCTTG
-20   AAGCCTGTCC TTGGAGAAAT  ATG

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Figure 1.1: The sequence of the proximal mouse GnRH receptor promoter. The two GSE homologues are in bold and underlined. The CRE is underlined, and the AP-1 site and the GRAS element are in bold. The numbers are relative to the translation start site.

1.6.2) Previous work completed in this laboratory

In her M.Sc. thesis, Carmen Pheiffer presented a gel mobility shift assay result showing a major DNA-protein complex formed between the proximal GSE (site 1) and α T3-1 nuclear extracts. A second, minor complex with higher mobility was also obtained, but this complex appeared to vary in intensity and mobility. Thus, early evidence from our laboratory already indicated that at least one, and possibly two, nuclear proteins from α T3-1 cells recognized site 1.

She also investigated the transcriptional activity of the proximal 560 base pairs of the mouse GnRH receptor promoter in various cell lines. The highest activity was observed in α T3-1 cells and in GH₃ pituitary somatolactotrope cells, with virtually no activity in COS-1 monkey kidney cells. Promoter activity in α T3-1 cells was significantly increased when a SF-1 expression construct was co-transfected with the promoter construct. The pituitary-specific activity of the promoter, and the positive effect of SF-1 protein on promoter activity, was therefore established (Pheiffer, 1998).

Gustav Styger investigated the role of sites 1 and 2 in the regulation of basal promoter activity in α T3-1 cells. In addition, he investigated whether the -590 to -2 (relative to the translation start site) promoter fragment was responsive to stimulation of the PKA pathway, and whether this effect was mediated via site 1 (Styger, M.Sc. thesis, 2001). For these experiments, he used a different promoter reporter construct than what was originally prepared by Clay *et al.*. The latter construct was prepared using a restriction site in the promoter fragment that overlaps site 1 (Clay *et al.*, 1995). Consequently, this construct does not contain the wild-type flanking sequences down-stream of site 1. For the purposes of Styger's experiments, a new cloning site was created downstream of site 1, thereby preserving the original flanking sequences. He found that the basal transcriptional activity of the promoter was not influenced, compared to the wild-type, upon independent mutation of either site 1 or site 2. However, promoter activity was significantly reduced when both sites were mutated simultaneously. Furthermore, his results indicated that promoter activity was enhanced in response to forskolin, a PKA pathway activator. This stimulation was lost upon mutation of site 1. The role of site 2 in the response to PKA was not investigated (Styger, 2001). Together, these results provided strong evidence for an important functional role for both site 1 and site 2.

1.6.3) The aim of this study

Originally, this project set out to determine whether or not SF-1 protein from α T3-1 cells binds to site 1 and site 2 in the mouse GnRH receptor promoter. This was to be confirmed by using anti-SF-1 antibodies in gel mobility shift assays. In addition, by using various other specific antibodies, attempts were to be made to identify the other α T3-1 nuclear proteins that formed complexes with the two sites. Another aim of this project was to determine whether the stimulatory effect that the PKA pathway had on promoter activity was due to a direct effect on the promoter, or indirectly due to an increase in SF-1 expression levels that could ultimately lead to increased promoter activity.

Chapter 2: Materials and Methods

2.1) Oligonucleotides

2.1.1) Oligonucleotide sequences

All oligonucleotides were synthesized and provided by the DNA synthesizing facility of the University of Cape Town. The following double-stranded oligonucleotides were used:

site 1: 5' GAA GCC TGT CCT TGG AGA AA 3'
 3' CTT CGG ACA GGA ACC TCT TT GG 5'

site 1 mutated: 5' GATC CCT GTT ttT GGA GAA 3'
 3' GGA CAA aaA CCT CTT CTAG 5'

site 2: 5' TAC ACT TGG CCT TCA GGA GG 3'
 3' ATG TGA ACC GGA AGT CCT CCGG 5'

site 2 mutated: 5' AGGGGC TACGGT TACACT gcagCT TCAGGA GGGCTT GGC 3'
 3' TCCCCG ATGCCA ATGTGA cgtcGA AGTCCT CCCGAA CCG 5'

SF-1 binding site: 5' GATC GAT GAC CTT GGG AGA 3'
(rat aromatase promoter) 3' CTA CTG GAA CCC TCT CTAG 5'

Nur77 binding site: 5' TGC CGG GAA GGT CAA AGT CCC GCG 3'
(NurRE) 3' ACG GCC CTT CCA GTT TCA GGG CGC 5'

The wild-type GnRH receptor promoter oligonucleotides were designed from the sequence of the mouse GnRH receptor 5' flanking region, as published by Clay *et al.*, 1995. Site 1 was mutated by substituting nucleotides found to be essential for SF-1 binding (Horn *et al.*, 1992). The two strands of the oligonucleotide representing the mutated site 2 were also used as primers in the PCR mutagenesis of site 2 in the -590 to -2 promoter fragment (Styger, 2001), and the mutated base-pairs

constitute a new *Pst*I restriction site. The sequence of the oligonucleotide containing the SF-1 binding site from the rat aromatase promoter was obtained from Lynch *et al*, 1993, and the sequence of the NurRE from the human POMC promoter was obtained from Okabe *et al*, 1998.

2.1.2) Annealing of single-stranded oligonucleotides

Complementary single-strand oligonucleotides were mixed in equimolar ratios (10 µl of a 1 mM solution in H₂O of each) and incubated as follows: 88 °C for 2 minutes, 65 °C for 10 minutes, 37 °C for 10 minutes and 25 °C for 5 minutes. Finally, the annealing mix was chilled on ice. The double-stranded oligonucleotides were aliquotted and stored at –20 °C. The integrity of the double-stranded oligonucleotides were checked by electrophoresis on a 2% (w/v) agarose (Whitehead Scientific) 1 X TAE (Sambrook *et al*, 1989) gel, and their mobility was compared to that of the single-stranded oligonucleotides.

2.2) Antibodies, antisera, protein markers, reagents

Specific antiserum (200 µg IgG in 83 µl Tris-Glycine buffer) against the DNA binding domain of SF-1 was purchased from Upstate Biotechnology, Lake Placid, New York. Antiserum against whole SF-1 protein was a kind gift from Dr. Ken Morohashi, of the Department of Molecular Biology at the Graduate School of Medical Science of the Kyushu University, Japan. Antiserum against Nur77 used in gel mobility shift assays was a kind gift from Dr. Thomas Perlmann, of the Karolinska Institute in Stockholm, Sweden. Purified antibody against Nur77 used in Western blot analyses was purchased from Geneka Biotechnology, Montreal, Quebec, Canada, along with nuclear extracts from CCRF-CEM human T-cell leukemia cells (5 µg/µl), as a positive control for the presence of Nur77 protein (Geneka Biotechnology product catalogue, 2000 - 2001). The purified antibody against COUP-TF (2 mg/ml) was purchased from Whitehead Scientific, South Africa, as agents for Santa Cruz Biotechnology, USA. Rabbit pre-immune serum (70 mg/ml) was a kind gift from Prof. D. U. Bellstedt, of the Department of Biochemistry, University of Stellenbosch. Rabbit peroxidase

anti-peroxidase (PAP) was purchased from Sigma. Secondary goat anti-rabbit serum was either purchased from Sigma or prepared by Dr. Amanda Swart, of the Department of Biochemistry, University of Stellenbosch. Molecular weight protein standards (14 – 220 kDa, \pm 1 mg/ml of each standard, # RPN756) were purchased from AEC Amersham. These standards are commonly known as Rainbow Markers, as they have been derivatized to each have a distinct colour.

The following general reagents were used: KCl and EDTA (di-sodium salt) (Saarchem UnivAR); NaHCO_3 , NaCl and NaOH (Saarchem UniLAB); SDS (BDH); HEPES (BDH, biochemical grade); KH_2PO_4 , glacial acetic acid and methanol (BDH AnalaR); Na_2PO_4 (BDH general purpose reagent); MgCl_2 (Riedel-de Haën, analytical grade); dithiothreitol (DTT) (Roche); phenylmethane sulfonyl fluoride (PMSF) (Merck, biochemistry grade); tryptone powder (Merck Biolab), tris(hydroxymethyl)aminomethane and sodium citrate (Merck, reagent grade); glycerol (Holpro-Lavasz); yeast extract (USB); agar (Biolab); glucose (Synthon Fine Chemicals); ethidium bromide (AEC Amersham).

Reagents that were used for specific procedures are mentioned under the relevant sections.

2.3) Plasmids and plasmid preparations

2.3.1) Plasmids

The full-length 2 kb SF-1 cDNA (from human origin) cloned into the pCMV-SF-1 expression vector was a kind gift from Dr Keith Parker, of the University of Texas Southwestern Medical Center (refer to Ikeda *et al*, 1993 for sequence). The full-length cDNA was excised by EcoRI restriction enzyme digestion and cloned into the pSPT19 vector (obtained from Roche Molecular Biochemicals), orientated such that transcription in the sense direction takes place from the T7 promoter. The full-length 2,1 kb human fibroblast cytoplasmic β -actin cDNA (clone pHF- β A-1) was originally cloned into the Okayama-Berg expression vector (Gunning *et al*, 1983) and was a

kind gift from Sihaam Boolay, of the Department of Biochemistry, University of Cape Town. Both vectors have a selective marker for ampicillin resistance.

2.3.2) Preparation of electrocompetent cells

Electroporation was performed with a Savant GTF100 gene transformer/electroporator, and the protocols for the preparation and transformation of electrocompetent cells, as described in the Savant Instruction Manual, were strictly followed.

E. coli JM109 bacterial cells were grown in LB medium (Sambrook *et al*, 1989) to an optical density at 550 nm of 0,8 and chilled on ice. All the following steps were performed at 4 °C. Cells were pelleted by centrifugation at 25000g for 15 minutes. They were washed twice by resuspension in 0,4 culture volumes cold sterile distilled water and centrifugation at 2500g for 15 minutes. The wash steps were repeated twice with ice-cold sterile 10% glycerol. Finally, the cells were resuspended in 10% glycerol to an optical density at 550 nm of 100 to 200, aliquotted and stored at -70 °C.

2.3.3) Electroporation

Electrocompetent cells were diluted 1:1 in ice-cold 10% glycerol and 40 µl chilled cell suspension was added to 1 ng of plasmid DNA in a chilled microcentrifuge tube. This was transferred to a chilled electroporation cuvette and subjected to an electric pulse. The transformed cells were immediately mixed with 1 ml of cold SOC medium (Sambrook *et al*, 1989) and incubated for 1 hour at 37 °C with constant agitation. The cells were plated out at different dilutions on LB agar plates containing 50 µg/ml ampicillin (Sigma), and allowed to grow overnight at 37 °C.

2.3.4) Selection of clones and preparation of glycerol stocks

Positive clones, as selected for by ampicillin, were picked off the LB agar plates with a sterile tooth-pick and suspended in 20 µl sterile water. The cell suspension was vortexed and 400 µl LB medium containing 50 µg/ml ampicillin was added. The cells were grown overnight at 37 °C while shaking at 200 - 225 rpm. Afterwards, 100 µl 80% (v/v) glycerol was added, the suspension was vortexed and stored at -70 °C.

2.3.5) Plasmid preparations

Mid-scale plasmid preparations were performed with the Promega Wizard Midiprep kit. The plasmids were prepared according to the protocol provided, with the following modification: The plasmid DNA was eluted by incubating the column with TE buffer, pH 8.0 (Sambrook *et al*, 1989) (pre-heated to 65 °C), for 15 minutes, followed by centrifugation at 10000g for 20 seconds.

2.4) Cell culture conditions

αT3-1 mouse pituitary precursor gonadotrope cells were kindly supplied by Dr Pamela Mellon, from the University of California, San Diego, California. The cells were grown in Dulbecco's modified Eagle Medium (GibcoBRL) (4,5 g/l glucose) with 10% fetal calf serum (Highveld Biologicals), 50 IU/ml penicillin and 50 µg/ml streptomycin (GibcoBRL). The cells were maintained in 75 cm² cell culture flasks (purchased from B & M Scientific, South Africa, as agents for TPP, Switzerland) at 37 °C with 90% humidity and 5% CO₂.

The cells were trypsinized by incubating for 2 to 3 minutes in 3 ml 0,25% trypsin in calcium- and magnesium-free phosphate-buffered saline (Highveld Biologicals). Afterwards, the flasks were tapped against the side to dislodge the cells. The cells were subsequently split in a 1:5 ratio.

Two batches of nuclear extracts were prepared during the course of the experiments, one each on 8 September 1999 and 15 June 2000. While culturing the cells for the second extraction, great care was taken to ensure that the cells were not passaged more than 10 times, whereas for the first extraction, the number of passages was not closely monitored, and could have exceeded 10.

2.5) Nuclear extract preparation

Twenty 75cm² flasks of confluent α T3-1 cells were harvested with a rubber cell scraper in phosphate-buffered saline (PBS) (Sambrook *et al*, 1989). The entire extraction was performed at 4 °C. The cells were pelleted by centrifugation at 1000g for 10 minutes, and washed twice in 5 pellet volumes PBS. The cell pellet was resuspended in 5 pellet volumes Buffer A: 10 mM HEPES, pH 7,9; 1,5 mM MgCl₂; 10 mM KCl; 0,5 mM DTT and 0,05 mM PMSF, and the cells were incubated on ice for 20 minutes. The cells were pelleted again by centrifugation at 1000g for 10 minutes, resuspended in 2 pellet volumes Buffer A and lysed by ten strokes in a Dounce homogenizer. A crude nuclear pellet was obtained by centrifugation at 2000g for 10 minutes. This was resuspended in 4 pellet volumes Buffer A and centrifuged at 25000g for 20 minutes to obtain a nuclear pellet. The nuclei were resuspended in 500 μ l Buffer C: 20 mM HEPES, pH 7,9; 1,5 mM MgCl₂; 1 mM EDTA; 25% (v/v) glycerol; 0,5 mM DTT and 0,05 mM PMSF. 250 μ l 1 M KCl was added and the nuclei were lysed while gently rocking at 4 °C for 30 minutes. Subsequently, the insoluble nuclear debris was pelleted at 25000g for 30 minutes. The supernatant was dialysed for 3 X 2 hours in 200 volumes Buffer D (10 mM HEPES, pH 7,9; 5 mM MgCl₂; 50 mM KCl; 1 mM EDTA; 10% (v/v) glycerol; 3 mM DTT and 0,3 mM PMSF). Before use, 20 cm of dialysis tube was boiled for 10 minutes in a large volume of 2% NaHCO₃ and 1 mM EDTA, autoclaved in 1 mM EDTA and rinsed in distilled autoclaved water. The dialysed nuclear extracts were stored in aliquots at -70 °C.

The extracts prepared on 8 September 1999 and 15 June 2000 had a protein concentration of 0,9 mg/ml and 1,2 mg/ml, respectively.

2.6) *In vitro* transcription-translation of proteins

This was done with the Promega TnT® Quick Coupled Transcription/Translation system, according to the protocol provided. 2 µg of supercoiled plasmid DNA was used per 50 µl reaction mix and the transcription-translation mix was incubated for 2 hours at 30 °C. A mock reaction was performed in parallel with the transcription-translation reaction, where the cDNA was replaced with nuclease-free water ("no cDNA control"). The reaction products were stored in 5 µl aliquots at -80 °C.

2.7) Radioactive labelling

2.7.1) Labelling of double-stranded oligonucleotides with polynucleotide kinase (PNK)

Labelling was performed according to the protocol provided with the PNK (Roche Molecular Biochemicals). 10 picomoles of double-stranded oligonucleotide was mixed with 2 µl 10 X phosphorylation buffer (provided with the enzyme) and 20 pmol [γ -³²P] ATP (AEC Amersham). 10 units PNK and water to a final volume of 20 µl were added, followed by a 30 minute incubation at 37 °C. The reaction was stopped by cooling on ice, followed by addition of 1 µl 0,5 M EDTA and 79 µl TE buffer. The labelling mix was passed through a 1ml G50 Sephadex (Pharmacia) spin column (equilibrated with TE buffer) to separate unincorporated nucleotides. The specific activity of the labelled DNA ranged from 10⁷ to 10⁹ dpm/µg DNA, and was independent of the oligonucleotide being labelled.

2.7.2) Labelling of cDNA probes for Northern hybridization

Labelling was done with the Megaprime DNA Labelling System (Amersham Pharmacia Biotech). 25 ng linear probe, 5 µl primer and water to a final volume of 33 µl were mixed. This was boiled in a waterbath for 5 minutes, followed by the addition of 10 µl 10 X labelling buffer, 50 µCi [α -³²P]-dCTP (AEC Amersham) and 2 µl (2 units) Klenow enzyme. The labelling mix was incubated for 1 hour at 37 °C, the volume made up to 100 µl with TE buffer and the labelling mix passed through a 1 ml G50 Sephadex spin column (equilibrated with TE buffer) to separate unincorporated

nucleotides. The labelling efficiency of the SF-1 cDNA probe was approximately 85%, with a specific activity of $6,3 \times 10^8$ cpm/ μ g DNA, while that of the β -actin probe was approximately 60%, with a specific activity of $3,5 \times 10^8$ cpm/ μ g DNA.

2.8) Gel mobility shift assays

The assay protocol was adapted from the method described by Horn *et al*, 1992. Assays were done in final buffer concentrations of 100 mM NaCl, 7.5 mM HEPES (pH 7.9), 10 mM Tris-HCl, 8% (v/v) glycerol, 1.25 mM $MgCl_2$, 1mM EDTA (pH 8.0), 0.25 mM EGTA (pH 7.0), 0.675 mM PMSF and 3.5 mM DTT. 0.5 μ g of poly(dIdC) (Roche) was added as non-specific DNA competitor to each assay incubation, as well as 6 μ g of bovine serum albumin (BSA) (Roche, molecular biology grade). The final incubation volume was 5 μ l. All incubations were done with 1 μ l (0,1 picomoles) radiolabelled double-stranded oligonucleotide probe (150 000 to 250 000 cpm) and 1,5 μ l (1 to 2 μ g) of either α T3-1 nuclear extracts, *in vitro* transcribed-translated protein (diluted 1:7 in TE buffer) or CCRF-CEM nuclear extracts (diluted 1:4 in TE buffer). The proteins were incubated for 10 minutes on ice in the absence or presence of various antibodies, after which radiolabelled probe was added, followed by an incubation for 10 minutes at room temperature. For antibody assays, 1 μ l of undiluted antibody or antiserum was added, except in the case of rabbit pre-immune serum, where 1 μ l of a 1:28 dilution was added. For competition assays, an excess of cold competitor oligonucleotides was mixed with the radiolabelled probe before adding it to the assay incubation.

Polyacrylamide (4% of 29:1 acrylamide:bisacrylamide) gels were electrophoresed using a BioRad Protean ® II xi gel apparatus. The acrylamide:bisacrylamide mix was prepared by dissolving 14,5 g ultrapure acrylamide (USB) and 0,5 g N,N'-methylenediacrylamide (bisacrylamide, Merck, electrophoresis grade) in sterile deionized water to a final volume of 50 ml. This was passed once through a Whatman no. 2 filter. Gels were poured and allowed to set overnight at 4°C. Before the actual electrophoresis, the gels were pre-electrophoresed at 100 V for 2 hours in 1 X TAE buffer.

Assay incubations were loaded onto the gel and the complexes resolved by electrophoresis at 100V for 3 ½ hours in fresh 1 X TAE buffer. The gels were subsequently transferred to blotting paper, dried by vacuum and exposed to Hyperfilm (Amersham) at -80°C.

2.9) Northern blot analysis

2.9.1) Preparation of DNA probes

The membrane was probed for SF-1 RNA by using the full-length (2 kb) human SF-1 cDNA, excised from pSPT-19 by EcoRI restriction enzyme digestion. The β -actin probe was 1,9 kilobasepairs of human fibroblast cytoplasmic β -actin cDNA, excised from the Okayama-Berg expression vector by BamHI restriction enzyme digestion. Both restriction enzymes were purchased from Amersham. The cDNA fragments were excised overnight at 37 °C in the appropriate enzyme buffer, as recommended by the manufacturer. Upon completion, the reaction mixes were electrophoresed on a 1% (w/v) low melting point agarose (Whitehead Scientific) gel at 100V in 1 X TAE buffer for 90 minutes. The linear probes of desired length were extracted from the agarose with the NucleoSpin Extract kit (Macherey-Nagel), according to the protocol provided.

2.9.2) α T3-1 cell incubation and RNA isolation

α T3-1 cells were plated out in petri dishes (10 cm diameter) at a density of 2×10^6 cells per dish. The cells were grown to confluency and fresh medium plus fetal calf serum added. Forskolin (Sigma) was dissolved in dimethylsulfoxide (DMSO) (Merck, synthesis grade) to a stock concentration of 10 mM, and the cells were incubated for approximately 16 hours in the absence or presence of various concentrations of forskolin, in the presence of serum. The final concentration of DMSO in the medium did not exceed 1% (v/v). Subsequently, the medium was removed, 1 ml TRI reagent (Sigma) was added to each dish and the cells were harvested with an RNase-free rubber cell scraper. The harvested cells were then transferred to microcentrifuge tubes. Total α T3-1 RNA was isolated by G. Styger with TRI reagent, according to the protocol provided (Styger, 2001). The

final volume of RNA was 50 µl per dish, and the concentration of the isolated RNA ranged from 5 to 6 µg/µl. The RNA yield from each dish ranged between 250 and 300 µg. RNA aliquots were stored at -80 °C. The purity of the RNA was determined by calculating the A_{260}/A_{280} ratio, and the integrity was tested on a 1% denaturing formaldehyde agarose gel. 10µg of each sample was incubated with 1 volume formaldehyde gel loading mix and 5 µg ethidium bromide (0,5 µl of a 10 mg/ml solution) for 15 minutes at 55 °C. Samples were electrophoresed for 2 hours and 15 minutes at 65V in 1 X MOPS buffer.

It is important to note that the α T3-1 cells from which the total RNA was isolated were cultured under the same conditions as the cells used for the first nuclear protein extraction, i.e. the number of passages that the cells had undergone could have exceeded 10.

2.9.3) Electrophoresis and membrane transfer

Water used for RNA work was treated with 0,1% (v/v) diethyl pyrocarbonate (DEPC) (Sigma) and autoclaved twice. All equipment was rendered RNase-free by soaking for 2 hours in 0,5 M NaOH and rinsed with DEPC-treated H₂O. 20 µg RNA of each sample was incubated with 1 volume formaldehyde gel loading mix for 15 minutes at 55 °C. The samples were separated on a 1% denaturing formaldehyde agarose gel in 1 X MOPS electrophoresis buffer at 65 V for 2 hours and 15 minutes.

The separated RNA was transferred overnight onto Hybond N⁺ membrane (Amersham Pharmacia Biotech) by means of capillary blotting in 20 X SSC buffer (Sambrook *et al*, 1989). After transfer, the membrane was rinsed in 2 X SSC buffer and blotted on filter paper to dry. The RNA was subsequently cross-linked to the membrane by ultra-violet light of 312 nm for 12 seconds, using an Ultraviolet Crosslinker (Amersham).

The membrane was prepared for hybridization by incubating in 20 ml DIG EasyHyb solution (Roche) at 50 °C for 30 minutes. 100 µl of ^{32}P -labelled double-stranded DNA probe ($\pm 1,5 \times 10^7$ cpm) was denatured at 95 °C for 10 minutes, to separate the strands. Hybridization was performed overnight at 50 °C in 15 ml DIG EasyHyb solution. After hybridization was completed, the membrane was washed twice for 5 minutes at room temperature in 2 X SSC buffer containing 0,1% SDS. The membrane was subsequently washed twice for 15 minutes at 50 °C in 0,1 X SSC buffer containing 0,1% SDS. The duration of the second washes depended on the background signal remaining on the membrane, which was roughly determined by using a hand-held Geiger counter. The washed membrane was wrapped in cling-wrap and exposed to Hyperfilm (Amersham) at -80 °C for 2 days. Later, the membrane was stripped by pouring boiling 0,5% SDS over it and allowing it to cool at room temperature for 2 to 3 hours. The membrane was subsequently probed for β -actin mRNA, as described above, and exposed to Hyperfilm for 2 hours.

2.10) SDS-PAGE and Western blot analysis

Protein samples contained either 5 µl undiluted *in vitro* transcribed-translated protein or varying amounts of nuclear extracts, as specified in the legends of relevant figures. The samples were boiled in 1 X SDS sample buffer (Sambrook *et al*, 1989) containing 5% (v/v) β -mercaptoethanol (Merck). Proteins were separated on an 8% SDS-PAGE gel (Sambrook *et al*, 1989), using a BioRad Protean® II xi gel apparatus, for 5 hours at 120 V in 1 X SDS gel running buffer (25 mM TRIS-HCl, 250 mM glycine (Merck, reagent grade), 0,1% SDS, pH 8,3). 5 µl Rainbow Markers were also loaded on the gel. The separated proteins were transferred onto a nitrocellulose membrane (Schleicher-Schuell) by electroblotting for 16 hours at 120 mA in 1 X transfer buffer (25 mM TRIS-HCl, 192 mM glycine (Saarchem UnivAR), 20% (v/v) methanol, pH 8,3).

2.10.1) Western blot for SF-1 protein

The electroblotting apparatus was disassembled and the membrane was blocked with casein buffer at room temperature for 1 hour. No wash steps were performed after the transfer. The membrane was incubated overnight with a 1: 5000 dilution of anti-SF-1 antibody (Upstate) or 1: 3000 dilution of anti-SF-1 antibody (Ken Morohashi) at 4 °C. Incubation with the secondary antibody (Dr. A. Swart) was performed at 37 °C for 1½ hours at 1:500 dilution. Finally, the membrane was incubated with peroxidase-anti-peroxidase antibody (PAP) (Sigma) at a 1:5000 dilution for 1½ hours at 37 °C. All antibody dilutions were made in casein buffer. Each antibody incubation was followed by 1 X 15 minute wash in PBS and 1 X 5 minute wash in PBS-0,1% (v/v) Tween at room temperature. Specific protein-antibody complexes were visualized by the ECL Detection (Amersham Pharmacia Biotech) method, according to the protocol provided with the product. Briefly, equal volumes of ECL Detection Solution 1 and 2 were mixed, poured onto the membrane and incubated for 60 seconds, after which the membrane was wrapped in cling-wrap and exposed to Hyperfilm at room temperature for approximately 4 minutes. The Rainbow Markers do not give a signal on the film during ECL detection. Therefore, the positions of the coloured marker bands on the membrane were marked on the film after the detection was completed.

2.10.2) Western blot for Nur77 protein

This experiment was performed as described for SF-1 protein above, but with the following differences. The membrane was blocked overnight in casein buffer at 4 °C and incubated with the primary antibody (Geneka Biotechnology) at a 1:2000 dilution for 2 h. Subsequently, it was incubated with the secondary antibody (Sigma) at a 1:1000 dilution, and with PAP at a dilution of 1:5000. Both these incubations were done for 90 minutes. All antibody dilutions were made in casein buffer and all incubation steps were carried out at 37 °C. Each incubation was followed by 3 wash steps of 5 minutes each in PBS. Before the ECL detection was performed, the membrane was washed once with PBS-0,1% (v/v) Tween for 15 minutes.

2.11) Buffers and mixes

2.11.1) Gel mobility shift assays

4% acrylamide gel mix (40 ml):

5,2 ml acrylamide solution (29:1 molar ratio acrylamide:bisacrylamide)

33,6 ml sterile deionized H₂O

0,4 ml 50 X TAE

68 µl TEMED (Merck, synthesis grade)

340 µl 10% (w/v) ammonium persulphate (BDH AnalaR)

Sample preparation (total volume per well 5 µl):

0,7 µl gel shift incubation buffer

0,5 µl poly(dIdC) (1 µg/µl) (Roche)

0,3 µl BSA (Roche, molecular biology grade) (20 mg/ml)

1,5 µl assay protein (described in section 8)

1 µl labelled DNA probe (0.1 pmol double-stranded oligonucleotide, ± 200 000 cpm)

and 1 µl antibody (described in section 8)

or 1 µl non-radiolabelled competitor oligonucleotide

2.11.2) RNA agarose electrophoresis

10 X MOPS buffer (pH 6.0):

41,86 g MOPS (Roche)

4,10 g sodium acetate (Saarchem UnivAR)

3,72 g EDTA (di-sodium salt)

Add DEPC-treated water to 1 litre

1% denaturing agarose formaldehyde gel (100 ml):

1 g agarose

10 ml 10 X MOPS

86 ml DEPC-treated H₂O

Boil, cool down and add 5,36 ml formaldehyde (Merck, reagent grade)

Formaldehyde gel loading solution:

63 μ l H₂O

51 μ l formaldehyde

48 μ l bromophenol blue in 50% glycerol (BDH)

48 μ l 10 X MOPS

2.11.3) Western blot

Casein buffer (500 ml):

4,5 g NaCl

2,5 g casein (BDH biochemical grade)

0,6 g Tris(hydroxymethyl)aminomethane

0,1 g thiomersal (preservative agent) (BDH general purpose reagent)

Dissolve the components, excluding the casein, in sterile deionized water. Set the pH at 7.6, add casein and stir overnight at 4 °C.

Chapter 3: Results

3.1: Nuclear proteins from α T3-1 cells recognize two sites in the mouse GnRH receptor promoter.

3.1.1) Gel mobility shift assays

Once a specific promoter region has been identified to contain a possible *cis*-regulatory element, the first step in investigating that element would be to determine whether or not proteins bind there. Once protein binding has been established, the next step would be to determine which of these DNA-protein interactions are specific, and to identify the proteins forming these specific complexes. This can sometimes be more complicated than initially thought, since a crucial part of the process involves educated guessing. However, identifying the proteins that bind to the *cis*-element of interest almost always gives a good indication of the function of this element in the context of the promoter.

In order to identify whether proteins bind to specific promoter elements, double-stranded oligonucleotides representing these elements can be designed. These can then be radioactively labelled and used as probes in gel mobility shift assays. The assay is based on the principle that molecules and molecular complexes migrate through a non-denaturing polyacrylamide gel according to their individual size, charge and conformation. A protein bound to a radiolabelled DNA fragment will significantly retard the migration of the fragment through the gel. The DNA is therefore "shifted" because of protein binding. The protein-DNA complex can be visualized on an autoradiograph due to the radiation from the DNA. The DNA binding specificity of the protein in a given complex can be determined by co-incubation with an excess of non-labelled DNA competitors. Such competitors can either be the same DNA fragment as the radiolabelled probe, or

they can represent other sites of interest. They can also be fragments that are similar to the probe, but that contain base pair substitutions, to test the relative importance of specific base pairs in protein binding.

Identification of proteins in DNA-protein complexes can be achieved by means of antibodies specifically targeted against specific proteins. One therefore needs to have candidate proteins that are likely to recognize the DNA sequence in the probe. Once an antibody recognizes a DNA-binding protein in an assay incubation, it will either inhibit the formation of the complex (when it is targeted against the DNA-binding domain of the protein) or it will bind to the entire complex and retard its migration even more. The latter is generally referred to as a "supershift". Apart from cross-reactivity sometimes occurring between members of nuclear protein families, antibody assays are quite reliable tools for the identification of proteins recognizing specific DNA sequences.

In this chapter, binding of *in vitro* transcribed-translated SF-1 and α T3-1 nuclear proteins to two sites in the mouse GnRH receptor promoter was investigated. The sites, the proximal one at position -15, relative to the translation start site (site 1), and the distal one at position -245 (site 2), were indicated through sequence analysis to be candidates for SF-1 binding. Radiolabelled oligonucleotide probes representing these two sites were used in gel mobility shift assays, together with *in vitro* transcribed-translated SF-1 and nuclear extracts from α T3-1 cells, prepared on 8 September 1999 (batch 1). Since the rabbit reticulocyte lysate in which the *in vitro* transcribed-translated SF-1 was synthesized contains a whole collection of cellular proteins, a parallel translation reaction was performed in the absence of SF-1 cDNA, and this was used as a negative control. An antibody directed against the DNA-binding domain of SF-1, purchased from Upstate Biotechnology, were used to identify the protein-DNA complexes containing SF-1 protein. This antibody binds to the DNA-binding domain, and would therefore prevent formation of a SF-1-DNA complex.

3.1.2) *In vitro* transcribed-translated SF-1 and α T3-1 nuclear proteins bind to site 1 and site 2.

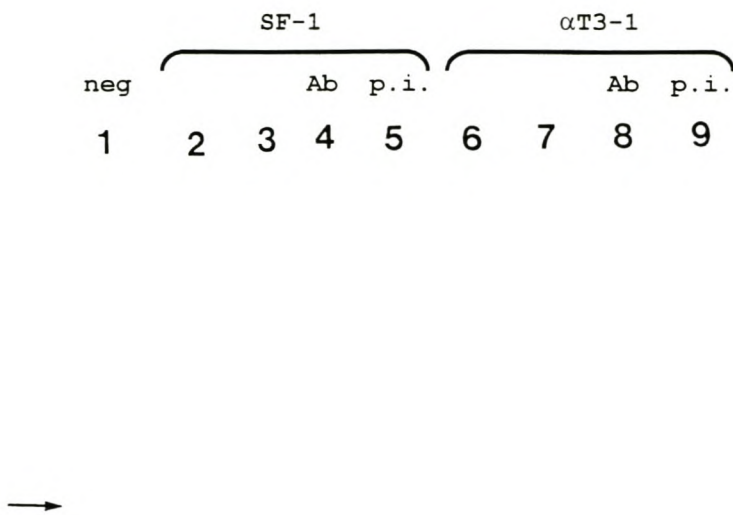


Figure 3.1: Autoradiograph of gel mobility shift assay performed with a probe representing site 1, located at -15 relative to the translation start site. Lane 1 shows the negative control (rabbit reticulocyte lysate without SF-1 cDNA). Lanes 2 to 5 contain *in vitro* transcribed-translated SF-1, lanes 6 to 9 contain α T3-1 nuclear extracts from batch 1. Lanes 2 and 3 were duplicate experiments, as were lanes 6 and 7. The arrow denotes the *in vitro* transcribed-translated SF-1 complex. Ab = anti-SF-1 antibody; p.i. = rabbit pre-immune serum. The band in lane 1 either represents a non-specific complex formed by a reticulocyte lysate protein, or a specific complex containing a lysate protein that is not SF-1.

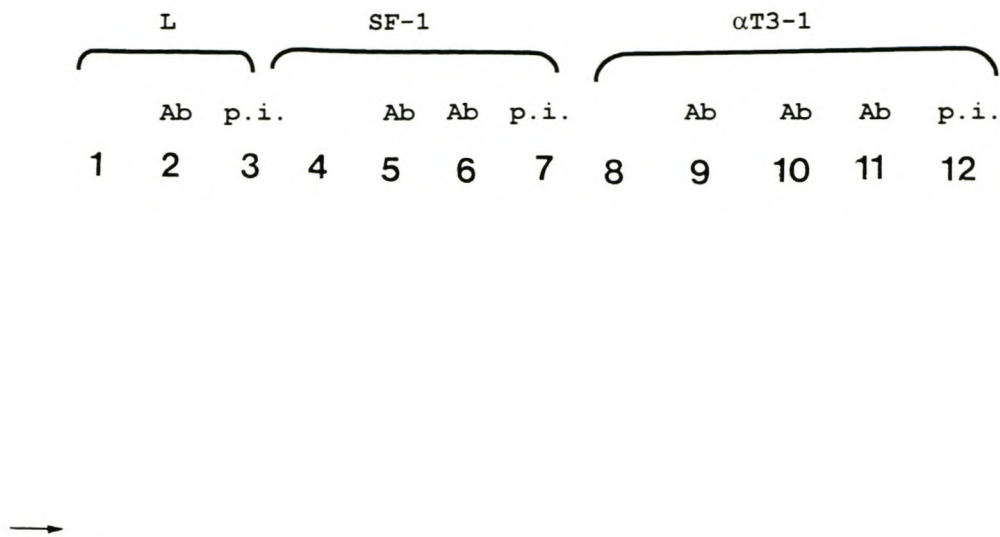


Figure 3.2: Autoradiograph of gel mobility shift assay performed with a probe representing site 2, located at -245 relative to the translation start site. Lanes 1 to 3 show the negative control (rabbit reticulocyte lysate minus SF-1 cDNA), with the effect of anti-SF-1 antibody on lysate proteins investigated in lane 2. Lanes 4 to 7 contain *in vitro* transcribed-translated SF-1, while lanes 8 to 12 contain αT3-1 nuclear extracts from batch 1. Lanes 5 and 6 each contain 2,4 μg anti-SF-1 antibody, whereas lanes 9 to 11 contain decreasing amounts of anti-SF-1 antibody (2,4 μg; 1,2 μg; 0,6 μg antibody, respectively). The arrow denotes the *in vitro* transcribed-translated SF-1 complex. L = reticulocyte lysate without cDNA; Ab = anti-SF-1 antibody; p.i = rabbit pre-immune serum.

Figure 3.1 shows that *in vitro* transcribed-translated SF-1 recognizes site 1. This is indicated by the presence of only one specific complex in lanes 2 and 3 (arrow), compared to the negative control in lane 1. The anti-SF-1 antibody prevents the formation of this complex in lane 4, confirming the protein in this complex to be SF-1. In lanes 6 and 7, no complex with similar mobility to that of the SF-1 complex is formed between the probe and α T3-1 nuclear proteins. In addition, the anti-SF-1 antibody did not prevent the formation of any of the complexes in lanes 6 and 7.

In figure 3.2 it can be seen that *in vitro* transcribed-translated SF-1 also recognizes and binds to site 2. This is shown by the formation of only one specific complex (indicated by an arrow) in lane 4, compared to the negative control in lane 1. This complex is lost upon incubation with anti-SF-1 antibody (lanes 5 and 6), but not with rabbit pre-immune serum (lane 7). The anti-SF-1 antibody is specific for SF-1 protein, since the antibody does not recognize any complexes formed between the probe and rabbit reticulocyte lysate (lane 2). Again, no complex with similar mobility to the SF-1 complex in lane 4 is formed between the probe and α T3-1 nuclear proteins. In addition, the anti-SF-1 antibody does not prevent the formation of any of the DNA-nuclear protein complexes.

Both figures 3.1 and 3.2 show that α T3-1 nuclear extracts contain several proteins that bind to sites 1 and 2. However, SF-1 protein does not appear to be present in any of the nuclear protein complexes. One interpretation could be that, assuming that functional SF-1 protein is present in α T3-1 nuclear extracts, it does not recognize the putative binding sites; hence, it plays no role in the regulation of the GnRH receptor via these *cis* elements. Alternatively, SF-1 protein could be present, but it could be non-functional due to a non-optimal extraction process. It could also be argued that SF-1 protein is contained in one of the complexes formed with nuclear extracts, possibly together with other nuclear proteins, and that the antibody is unable to recognize SF-1 as part of this multi-protein - DNA complex. On the other hand, it is also possible that SF-1 protein is not present in the nuclear extracts at all, or that it is present at undetectable levels. This could either be because

it was lost during the extraction process, or it might be that SF-1 is not expressed at detectable levels in the α T3-1 cells grown in this laboratory. It is conceivable that the expression levels of SF-1 could vary according to growth conditions.

3.1.3) *In vitro* transcribed-translated SF-1 and α T3-1 nuclear proteins bind to a confirmed SF-1 binding site.

In order to test the first of the several possibilities outlined in the previous section, the binding of *in vitro* transcribed-translated SF-1 and α T3-1 nuclear proteins to a confirmed SF-1 binding site was investigated. This was done by gel mobility shift assay, using a radiolabelled probe (SF-1 site) representing the SF-1 binding site from the promoter of the rat aromatase gene (Lynch *et al*, 1993). This experiment was based on the assumption that, if functional SF-1 protein is present in the α T3-1 nuclear extracts, then it should recognize a confirmed SF-1 binding site.

As can be seen in figure 3.3, one specific complex (arrow) was formed between *in vitro* transcribed-translated SF-1 and the SF-1 site (see lanes 2 and 3), but still no corresponding complex was formed between the probe and the nuclear extracts. The anti-SF-1 antibody did not prevent any of the DNA-nuclear extract complexes from forming (lane 8). This result strongly indicates that SF-1 is not present in the nuclear extracts, or that, if it is present, it is unable to recognize its confirmed binding site. Alternatively, the result could be explained by the possibility that SF-1 protein is contained in one of the DNA-nuclear protein complexes, but that some factor in the nuclear extracts is rendering the anti-SF-1 antibody unable to recognize its target protein.

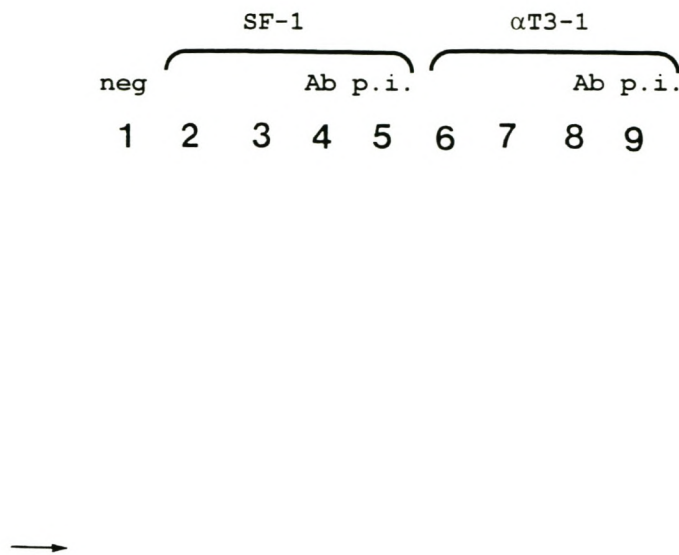


Figure 3.3: Autoradiograph of a gel mobility shift assay performed with the SF-1 probe. The negative control (refer to legends of figures 3.1 and 3.2) is shown in lane 1. Lanes 2 to 5 contain *in vitro* transcribed-translated SF-1, and lanes 6 to 9 contain αT3-1 nuclear extracts from batch 1. Lanes 2 and 3 were duplicate experiments, as were lanes 6 and 7. The arrow denotes the *in vitro* transcribed-translated SF-1 complex. Ab = anti-SF-1 antibody; p.i. = rabbit pre-immune serum.

3.1.4) Anti-SF-1 antibody recognizes SF-1 in the presence of α T3-1 nuclear proteins.

The last possibility described in section 3.1.3 was investigated by determining whether or not anti-SF-1 antibody recognizes *in vitro* transcribed-translated SF-1 in the presence of α T3-1 nuclear proteins. Therefore, the two protein mixtures were used together in a gel mobility shift assay, with the site 1 oligonucleotide arbitrarily chosen as probe.

This was a good control experiment, because the complexes formed between the site 1 probe and *in vitro* transcribed-translated SF-1 were the same as for the previous experiments performed with this probe (figure 3.4, lane 8; also refer to figure 3.1, lanes 2 and 3). The complexes formed between the probe and α T3-1 nuclear proteins were also the same as was previously obtained (figure 3.4, lane 7; also refer to figure 3.1, lanes 6 and 7). Figure 3.4 also shows that the complexes formed when the two sets of proteins were mixed were the sum of the complexes formed between the probe and the individual sets of proteins (compare lanes 5 and 6 with lane 7 and lane 8). It is clear that the presence of nuclear proteins did not prevent the formation of the band previously confirmed to contain *in vitro* transcribed-translated SF-1 (arrow). More importantly, it is clear that the anti-SF-1 antibody was able to recognize *in vitro* transcribed-translated SF-1 in the presence of α T3-1 nuclear proteins and prevent complex formation (figure 3.4, lanes 3 and 4). The anti-SF-1 antibody is therefore functional under all the conditions applied in the gel mobility shift assays, and one could assume that it would be able to detect SF-1-containing complexes. On the other hand, it is possible that SF-1 is contained in one of the DNA-nuclear protein complexes, as part of a multi-protein complex, and that it is shielded in such a manner that the antibody is unable to recognize it. The fact that no DNA-nuclear protein complexes disappear in the presence of this antibody strongly suggests that none of these complexes actually contain SF-1, and that other nuclear proteins are recognizing and binding to these putative SF-1 binding sites.

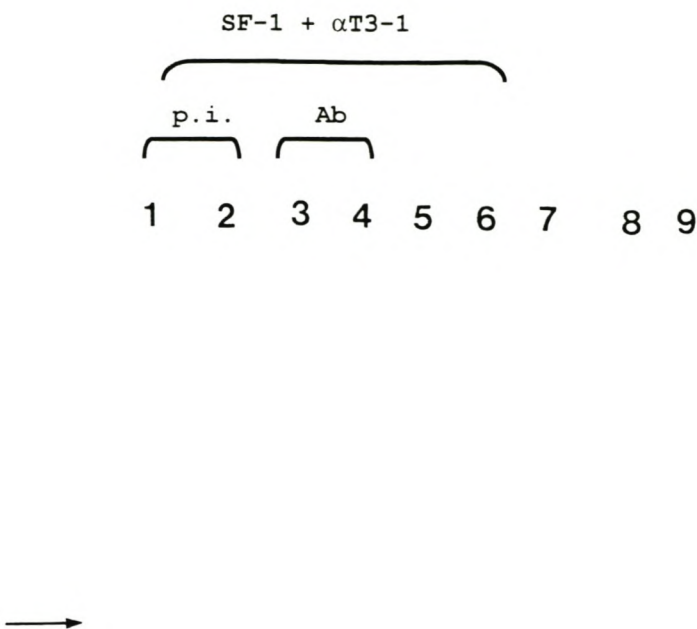


Figure 3.4: Autoradiograph of gel mobility shift assay performed with the site 1 probe, to investigate recognition of SF-1 protein by anti-SF-1 antibody in the presence of α T3-1 nuclear extracts from batch 1. In lanes 1 to 6, both *in vitro* transcribed-translated SF-1 and α T3-1 nuclear proteins were present. Lanes 7 contains α T3-1 nuclear extracts and lane 8 contains *in vitro* transcribed-translated SF-1. Lane 9 shows the negative control (refer to the legends of figures 3.1 and 3.2). Lanes 1 and 2 were duplicate experiments, as were lanes 3 and 4 and lanes 5 and 6. The arrow denotes the *in vitro* transcribed-translated SF-1 complex. Ab = anti-SF-1 antibody; p.i. = rabbit pre-immune serum.

3.2: Expression of SF-1 in α T3-1 cells

3.2.1) Western blot analysis using anti-SF-1 antibody against DNA binding domain

The gel mobility shift assay results presented in the previous chapter strongly suggested that SF-1 is not present in α T3-1 nuclear extracts. Alternatively, the possibility exists that SF-1 is present but inactive, or that it forms part of a multi-protein complex that cannot be recognized by the anti-SF-1 antibody. In order to investigate these possibilities further, it was essential to determine whether or not SF-1 protein was present in the α T3-1 nuclear extracts. This was done by Western blot analysis. This is a more definitive method than a gel mobility shift assay, since a Western blot does not depend on the activity of the target protein, and even inactive forms of the protein should be detected. Furthermore, the SDS polyacrylamide gel electrophoresis that precedes the blotting denatures the proteins, and would therefore dissociate multi-protein complexes into their monomeric subunits. A Western blot analysis is therefore an effective method of investigating several possibilities at once.

The anti-SF-1 antibody against the DNA-binding domain of SF-1, which was purchased from Upstate Biotechnology and was previously used in the gel mobility shift assays, was used as primary antibody in this experiment. *In vitro* transcribed-translated SF-1 was used as a positive control, and the "no cDNA" control of the rabbit reticulocyte lysate system was used as a negative control. The sizes of the proteins visualized on the membrane were compared to the sizes of a mixture of known protein standards.

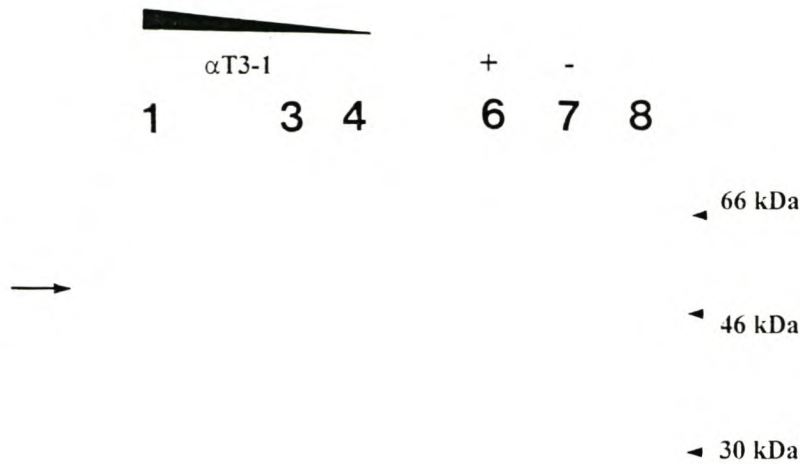


Figure 3.5: Western blot analysis performed with anti-SF-1 antibody (Upstate Biotechnology) on α T3-1 nuclear extracts from batch 1. Lanes 1, 3 and 4 contain 63, 54 and 36 μ g α T3-1 nuclear extract protein, respectively. Lane 6 shows *in vitro* transcribed-translated SF-1 as positive control (+) and lane 7 shows "no cDNA" rabbit reticulocyte lysate as negative control (-). Lanes 2 and 5 were left empty. Size markers were loaded in lane 8.

Lane 6 of figure 3.5 shows that *in vitro* transcribed-translated SF-1 protein contained a 53 kDa protein that reacted specifically with the anti-SF-1 antibody. This corresponds to the expected size of monomeric SF-1. However, no corresponding signal was detected in any of the lanes containing α T3-1 nuclear extracts (lanes 1, 3 and 4). Interestingly, a signal corresponding to a size of at least 66 kDa was observed in these lanes, but the identity of this protein is not known.

These results strongly suggest that SF-1 protein is not present in this batch of α T3-1 nuclear extracts. This was very encouraging, because it provided a simple explanation as to why no SF-1-DNA complexes could be detected in gel mobility shift assays with α T3-1 nuclear extracts and the

probes for site1, site 2 and the SF-1 site. Unfortunately, a fairly strong signal corresponding to 53 kDa was also observed in the negative control lane (figure 3.5, lane 7), indicating that the antibody was not specific enough to deliver a conclusive result. Therefore, the result needed to be confirmed, and the experiment was repeated with a different anti-SF-1 antibody. This antibody was a gift from Dr. Ken-ichirou Morohashi, and is directed against the whole SF-1 protein. Together with the first batch of α T3-1 nuclear extracts, a second batch was also tested for the presence of detectable levels of SF-1.

3.2.2 Western blot analysis using anti-SF-1 antibody against the whole protein

The result from this experiment, shown in figure 3.6, was significantly different from the previous experiment. Only a faint signal was detected in the lane with the negative control (lane 3), whereas a clear signal corresponding to 53 kDa was observed in the lane containing *in vitro* transcribed-translated SF-1 (lane 2). Most surprising, however, was the complete absence of any signal in the lane containing nuclear proteins from the first batch (lane 5), and the presence of a strong and unambiguous signal (arrow) in the lane containing proteins from the second batch of nuclear extracts (lane 7). This signal, also located at 53 kDa, showed that there was indeed SF-1 protein present in the second batch of α T3-1 nuclear extracts, but not in the first batch.

An interesting point arising from this result is that the levels of expression of SF-1 protein in α T3-1 cells appear to vary and could even be undetectable at certain times. This could have important implications for experiments performed in this cell line. Possible explanations for this variation in SF-1 protein levels will be discussed later.

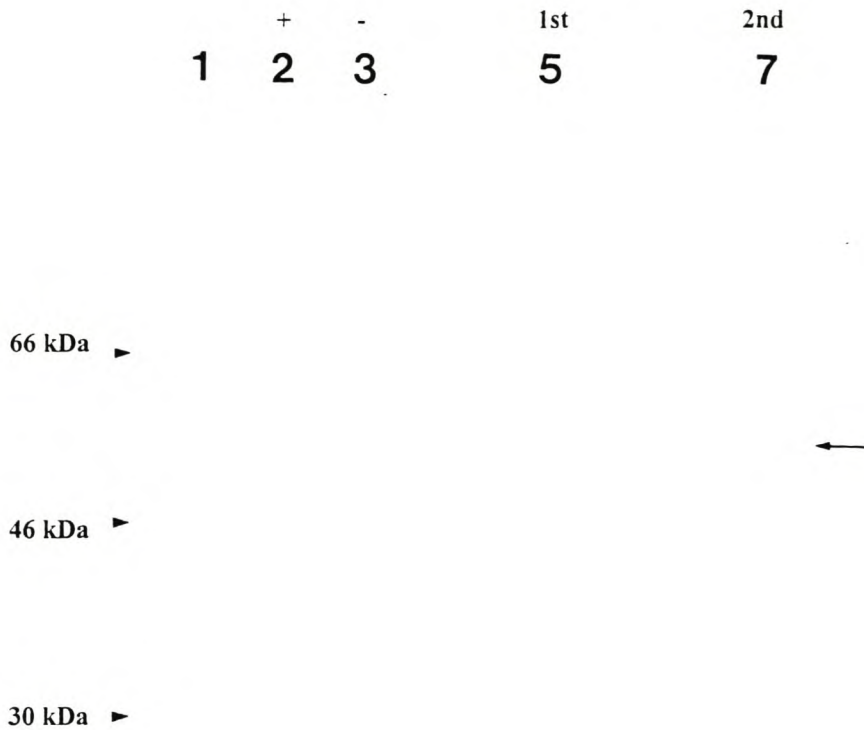


Figure 3.6: Western blot analysis performed with anti-SF-1 antiserum from Dr. K. Morohashi. Lane 2 shows *in vitro* transcribed-translated SF-1 as the positive control (+), and lane 3 shows "no cDNA" lysate as the negative control (-). Lanes 5 and 7 contain 55 μ g of α T3-1 nuclear extracts from the first (1st) and second (2nd) batches, respectively. Lanes 4 and 6 were left empty. The arrow denotes the SF-1 band. Size markers were loaded in lane 1.

The fact that the first batch of nuclear extracts did not contain any detectable SF-1 protein finally explained why no SF-1-DNA complexes were observed with α T3-1 nuclear extracts in the first set of gel mobility shift assays. It became clear that the assays had to be repeated with extracts that did contain SF-1, if any real conclusions about SF-1 binding to sites 1 and 2 were to be drawn. The Western blot result shown in figure 3.6 confirmed that there is SF-1 present in the second batch of α T3-1 nuclear extracts, and these were subsequently used in a new set of gel mobility shift assays.

3.3: SF-1 and Nur77 in α T3-1 nuclear extracts bind to sites 1 and 2

3.3.1) Identification of other nuclear proteins binding to the putative SF-1 sites

When gel mobility shift assays were performed with the second batch of α T3-1 nuclear extracts, several complexes were formed with both the site 1 and site 2 probes (see figures 3.7 and 3.8, lane 7), similar to the results obtained with the first batch of nuclear extracts. Some of these complexes appeared to be common to both probes, while each probe also formed distinct complexes. A comparison between the complexes formed by the two different batches of nuclear extracts revealed a new complex which was present in batch 2, but which was absent from batch 1. This can be seen when comparing lane 7 of both figures 3.7 and 3.8 with figure 3.1, lane 6 and figure 3.2, lane 8. This new complex was formed with both probes and had the same mobility as the SF-1 band formed by *in vitro* transcribed–translated SF-1 (see the asterices in lane 4 of figures 3.7 and 3.8). (Note that the SF-1 band in figure 3.7, lane 4 and figure 3.8, lane 6 is quite faint, but it can still clearly be seen in figure 3.7, lane 6 and figure 3.8, lane 4). Prior incubation of the second batch of nuclear extracts with anti-SF-1 antibody (Upstate Biotechnology) clearly prevented the formation of this complex (figures 3.7 and 3.8, lanes 8 and 9). This confirmed that the protein in this complex was SF-1 (see arrow labelled "SF-1"). Thus, SF-1 protein, present in the second batch of α T3-1 nuclear extracts, binds to both sites 1 and 2 in the mouse GnRH receptor promoter.

Specific anti-Nur77 antiserum (a gift from Dr. Thomas Perlmann) and anti-COUP-TF antibody (Santa Cruz Biotechnology) were also used in the gel mobility shift assays, in an attempt to identify the nuclear proteins in the other complexes. Surprisingly, anti-Nur77 antiserum supershifted a complex formed between nuclear extracts and the site 1 probe (figure 3.7, lanes 10 and 11), and it also supershifted a complex with similar mobility formed with the site 2 probe (figure 3.8, lanes 10 and 11, see arrows labelled "ss"). Nuclear extracts of the CCRF-CEM human T-cell leukemia cell line (Geneka Biotechnology), a positive control for the presence of Nur77 protein, formed a

complex with similar mobility to that of the putative Nur77 complex from α T3-1 nuclear extracts. Anti-Nur77 antiserum also supershifted this complex. This strongly indicated that the complex obtained with α T3-1 nuclear extracts contained Nur77 protein. When the nuclear extracts were incubated with anti-COUP-TF antibody, no complexes formed with either one of the two probes were supershifted.

Two other unidentified complexes (see figure 3.7, arrows labeled "a" and "b") were formed between the α T3-1 nuclear extracts and site 1, and one other between the α T3-1 nuclear extracts and site 2 (see figure 3.8, arrow labelled "c"). None of these complexes are influenced in any reproducible manner by any of the three specific antibodies, and it is possible that the proteins in these complexes bind non-specifically to the probes. The specificity of these complexes was investigated in the competition experiments, presented in section 3.4.

Therefore, these results show that SF-1 and Nur77 protein from α T3-1 nuclear extracts bind to both sites 1 and 2. COUP-TF protein does not appear to be present in any of the DNA-nuclear protein complexes.

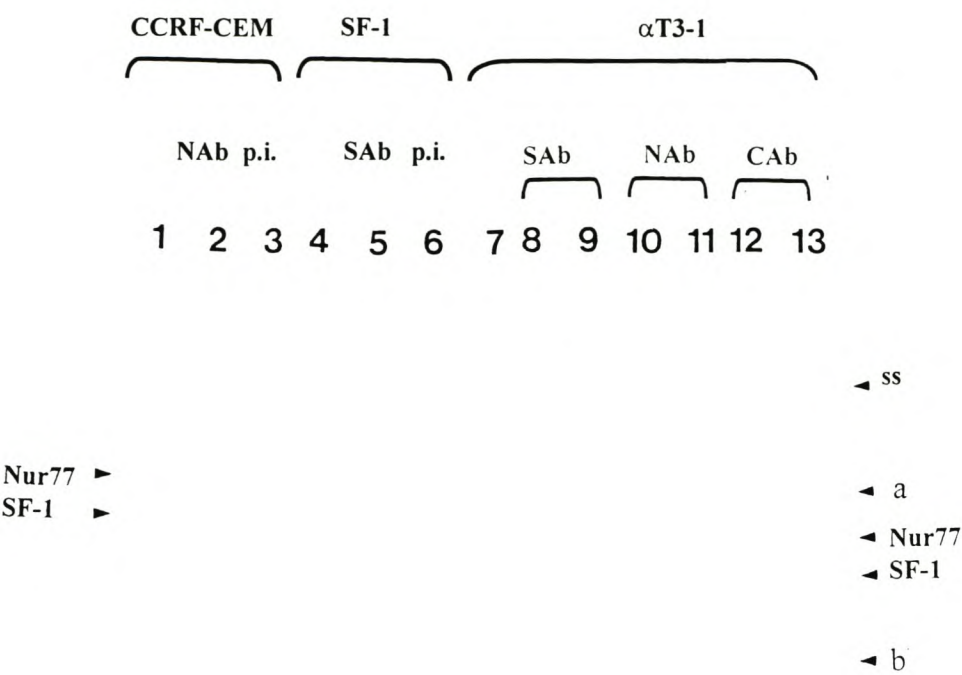


Figure 3.7: Autoradiograph of a gel mobility shift assay performed with the site 1 probe. Lanes 1 to 3 contain nuclear extracts from the CCRF-CEM human T-cell leukemia cell line (CCRF-CEM). Lanes 4 to 6 contain *in vitro* transcribed–translated SF-1 (SF-1), and lanes 7 to 13 contain αT3-1 nuclear extracts from batch 2 (αT3-1). Lanes 8 and 9 were duplicate experiments, as were lanes 10 and 11 and lanes 12 and 13. The arrows denote complexes containing SF-1 and Nur77 protein, respectively. NAb = anti-Nur77 antiserum (Perlmann); SAb = anti-SF-1 antibody (Upstate); CAb = anti-COUP-TF antibody (Santa Cruz); p.i. = rabbit pre-immune serum. Note that the two photographs in figure 3.7 were taken of the same autoradiograph, with a darker photograph taken of lanes 1 to 6 in order to enhance the visibility of the faint bands.

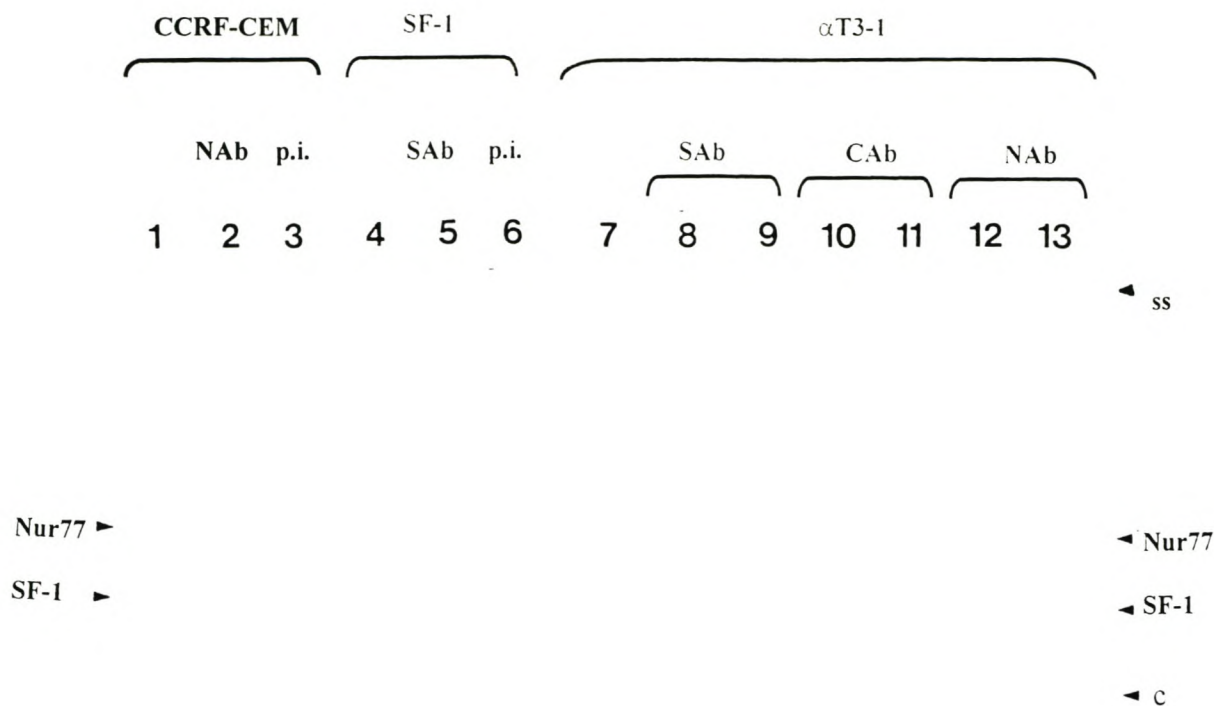


Figure 3.8: Autoradiograph of a gel mobility shift assay performed with the site 2 probe. Lanes 1 to 3 contain nuclear extracts from the CCRF-CEM human T-cell leukemia cell line (CCRF-CEM). Lanes 4 to 6 contain *in vitro* transcribed–translated SF-1 (SF-1), and lanes 7 to 13 contain α T3-1 nuclear extracts from batch 2 (α T3-1). Lanes 8 and 9 were duplicate experiments, as were lanes 10 and 11 and lanes 12 and 13. Arrows denote complexes containing SF-1 and Nur77 protein, respectively. NAb = anti-Nur77 antiserum (Perlmann); SAb = anti-SF-1 antibody (Upstate); CAb = anti-COUP-TF antibody (Santa Cruz); p.i. = rabbit pre-immune serum. Note that the three photographs in figure 3.8 were taken of the same autoradiograph, with a darker photograph taken of lanes 4 to 6, in order to enhance the visibility of the faint bands.

3.3.2) Nur77 protein is expressed in α T3-1 cells

From the Western blot analysis shown in figure 3.9, it can be seen that Nur77 protein is present in both batches of α T3-1 nuclear extracts investigated. However, the pattern of expression of Nur77 protein differed between the two batches. In the first batch, which did not contain detectable levels of SF-1, there are clearly two different forms of the Nur77 protein present (see lane 5). In the second batch of extracts, there is only one form (see lane 6). When the three protein bands in lanes 5 and 6 are compared, they appear to all have similar intensities. Hence, it can be concluded that the first batch of α T3-1 nuclear extracts contains approximately twice as much Nur77 protein as the second batch.

A very surprising result was therefore obtained, namely that Nur77 protein is expressed in α T3-1 cells. It was also shown that Nur77 protein binds to both the putative SF-1 binding sites in the mouse GnRH receptor promoter. This is, as far as could be ascertained, the first report of the presence of Nur77 protein in a gonadotrope cell line. The presence of Nur77 protein in gonadotropes has potentially very interesting implications, which will be discussed later.

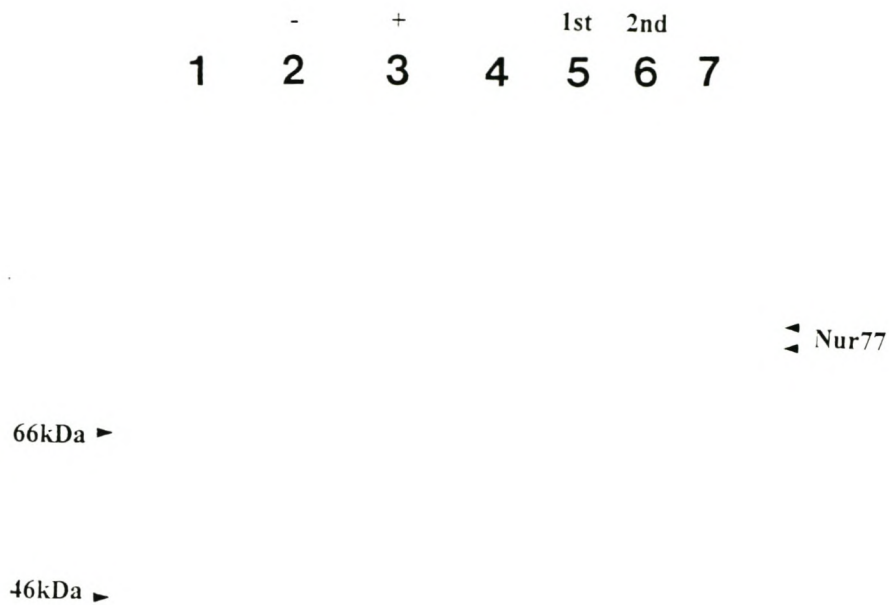


Figure 3.9: Western blot analysis performed on both batches of α T3-1 nuclear extracts using an anti-Nur77 antibody from Geneka Biotechnology. Lane 2 shows the negative control ("no cDNA" control rabbit reticulocyte lysate) (-) and lane 3 the positive control (20 μ g CCRF-CEM nuclear extracts) (+). Lanes 5 and 6 contain 35 μ g α T3-1 nuclear extracts of the first (1st) and second (2nd) batch, respectively. Lane 7 contain *in vitro* transcribed–translated SF-1, to indicate that the anti-Nur77 antibody does not cross-react with SF-1 protein. The Nur77 protein signals are indicated with arrows. Size markers were loaded in lane 1.

3.4: The relative affinities of SF-1 and Nur77 protein from α T3-1 cells for site 1 and site 2

3.4.1) Competition assays

The set of experiments presented in this section investigated the relative binding affinities of SF-1 and Nur77 protein from α T3-1 nuclear extracts for the site 1 and site 2 probes. In order to do this, the two probes were used as competitors for each other. An excess of non-radiolabelled probe was added to the assay incubations, to ascertain the relative affinities of the nuclear proteins for the radiolabelled probes. In addition to the two probes, four other double-stranded oligonucleotides were also used as competitors. Two of these represented sites 1 and 2, but with base-pair substitutions introduced into the core binding site. Two additional oligonucleotides, one containing the SF-1 binding site from the rat aromatase promoter (Lynch *et al*, 1993), and one containing the NurRE from the human POMC promoter (Okabe *et al*, 1998), were also used as competitors.

3.4.2) SF-1 and Nur77 protein have different DNA-binding specificities

From figure 3.10 it can be clearly seen that the site 1 competitor competes for the binding of SF-1 protein (lanes 2 to 4), but the mutated site 1 competitor does not (lanes 5 to 7). This is to be expected, since the residues that are essential for SF-1 binding (Horn *et al*, 1992) were substituted. Surprising, however, is the fact that, while the site 1 competitor competes for Nur77 binding, the mutated site 1 competitor also competes for Nur77 binding, albeit at a fairly high (125 fold) molar excess (lane 7). Note that this is not easily seen in the photograph, due to the intensity of the SF-1 complex in lane 7. However, it can be seen that, unlike in lane 5, there is no longer a Nur77 protein band above the SF-1 protein band in lane 7. Thus, Nur77 protein can still bind to a mutated or non-consensus SF-1 binding site, but with lower affinity than to wild-type site 1.

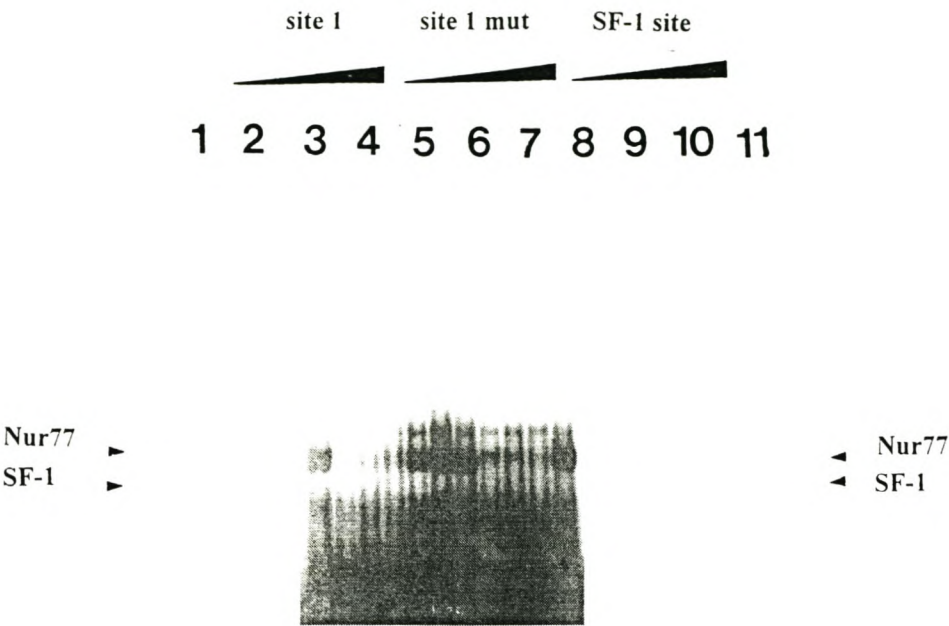


Figure 3.10: Autoradiograph of a gel mobility shift assay performed with α T3-1 nuclear extracts (batch 2) and the site 1 probe, in the absence and presence of varying concentrations of unlabelled competitor oligonucleotides. All the competitors were used at 5, 25 or 125 fold molar excess. In lanes 1 and 11, no competitors were added. Lanes 2 to 4 contain increasing amounts of site 1 competitor (site 1), lanes 5 to 7 contain mutated site 1 competitor (site 1 mut) and lanes 8 to 10 contain SF-1 binding site competitor (SF-1). The arrows denote the SF-1 and Nur77 protein complexes.

The SF-1 binding site competitor competes for the binding of both SF-1 and Nur77 protein. Full inhibition of SF-1 protein complex formation is achieved at 5 fold molar excess of the SF-1 binding site competitor (lane 8), and of Nur77 protein complex formation at 125 fold molar excess (lane 10). Therefore, it is clear that SF-1 and Nur77 protein have different DNA-binding specificities for the site 1, mutated site 1 and SF-1 competitors. To summarize the differences in affinity:

SF-1 protein: site 1 \approx SF-1 site \gggg mutated site 1

Nur77 protein: site 1 $>$ mutated site 1 \approx SF-1 site

Figure 3.11 shows that the site 2 competitor achieves almost complete inhibition of SF-1 protein complex formation with the site 1 probe at 25 fold molar excess (lane 3), and of Nur77 protein complex formation at 125 fold molar excess (lane 4). As was the case for the mutated site 1 competitor, the mutated site 2 competitor is unable to compete for SF-1 binding, but clearly competes for Nur77 binding (lane 5 to 7). Nur77 protein is therefore able to bind to both mutated sites 1 and 2, but SF-1 protein can no longer bind to either mutated site. The NurRE oligonucleotide competes for SF-1 protein binding at 25 fold molar excess (lane 9) and Nur77 protein binding at 125 fold molar excess (lane 10). The slowest migrating complex formed with the site 1 probe in all the gel mobility shift assays (complex a, refer to figure 3.7) appears to be a non-specific complex, since its intensity does not seem to be influenced (in any reproducible manner) by the presence of competitor oligonucleotides. This complex was therefore not further investigated. Figure 3.11 therefore shows that:

SF-1 protein: NurRE \approx site 2 \gggg mutated site 2

Nur77 protein: NurRE \approx site 2 \approx mutated site 2

The competitor experiments were subsequently repeated with site 2 as probe, and the full range of competitors was investigated.

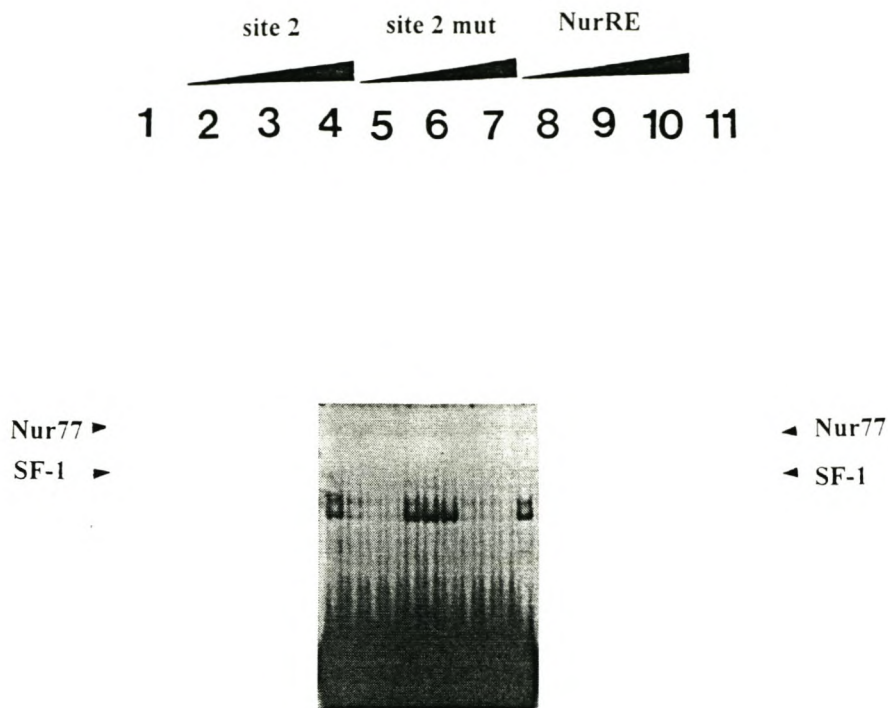


Figure 3.11: Autoradiograph of a gel mobility shift assay performed with α T3-1 nuclear extracts (batch 2) and the site 1 probe, in the absence and presence of varying concentrations of unlabelled competitor oligonucleotides. All the competitors were used at 5, 25 or 125 fold molar excess. In lanes 1 and 11, no competitors were added. Lanes 2 to 4 contain increasing amounts of site 2 competitor (site 2), lanes 5 to 7 contain mutated site 2 competitor (site 2 mut) and lanes 8 to 10 contain NurRE competitor (NurRE). Arrows denote the SF-1 and Nur77 protein complexes.

In figure 3.12, it can be seen that both the site 1 and SF-1 competitors already achieve complete competition for both SF-1 and Nur77 protein binding to the site 2 probe at 5 fold molar excess (lanes 2 and 9). As with the mutated site 2 competitor, the mutated site 1 competed for Nur77 binding, but not for SF-1 binding (lanes 6 to 8). The SF-1 binding site competitor competes for both SF-1 and Nur77 binding (lanes 9 to 11). Hence, the results of figure 3.12 are:

SF-1 protein: site 1 \approx SF-1 site \gggg mutated site 1

Nur77 protein: site 1 \approx SF-1 site \approx mutated site 1

Finally, in figure 3.13, it is once again clear that Nur77 protein does not discriminate between the wild-type and the mutated form of site 2 (lanes 5 to 7). The site 2 and NurRE competitors both fully compete for both SF-1 and Nur77 protein binding with site 2 at 5 fold molar excess (lanes 2 and 8).

Therefore, figure 3.13 indicates that:

SF-1 protein: site 2 \approx NurRE \gggg mutated site 2

Nur77 protein: site 2 \approx NurRE \approx mutated site 2

Comparing the results in figures 3.10 to 3.13, it can be seen that SF-1 protein has similar affinities for site 1 and site 2, and that this is also the case for Nur77 protein. Thus, to summarize:

SF-1 protein: site 1 \approx site 2 \approx SF-1 site \approx NurRE \gggg mutated site 1 \approx mutated site 2

Nur77 protein: site 1 \approx site 2 \approx mutated site 2 \approx NurRE $>$ mutated site 1 \approx SF-1 site

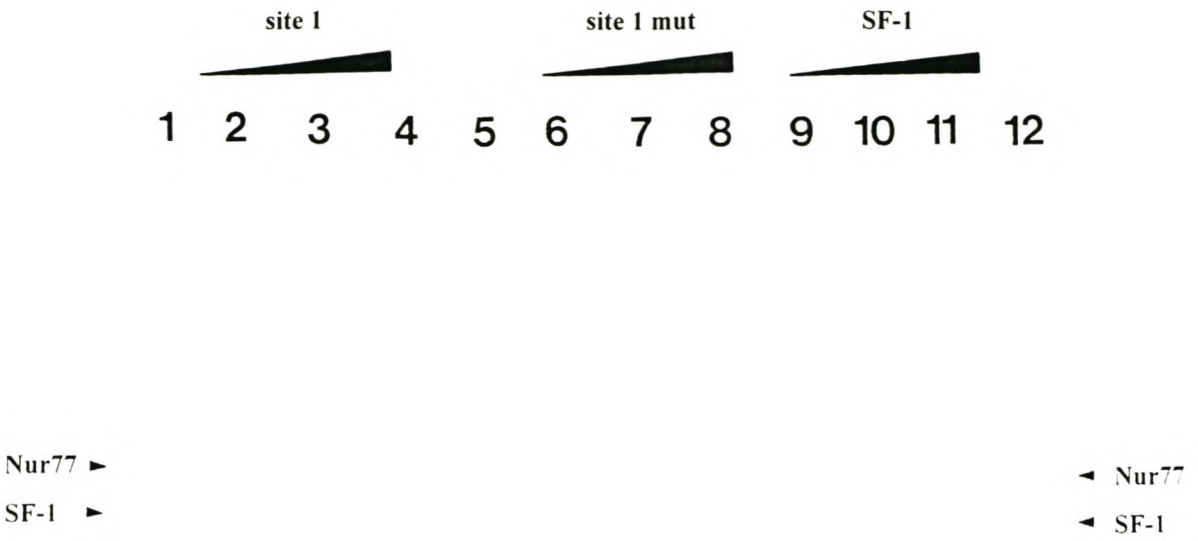


Figure 3.12: Autoradiograph of a gel mobility shift assay performed with α T3-1 nuclear extracts (batch 2) and the site 2 probe, in the absence and presence of varying concentrations of unlabelled competitor oligonucleotides. All the competitors were used at 5, 25 or 125 fold molar excess. In lanes 1, 5 and 12, no competitors were added. Lanes 2 to 4 contain increasing amounts of site 1 competitor (site 1), lanes 6 to 8 contain mutated site 1 competitor (site 1 mut) and lanes 9 to 11 contain SF-1 binding site competitor (SF-1). The arrows denote the SF-1 and Nur77 protein complexes. Note that the three photographs in this figure were taken of the same autoradiograph.

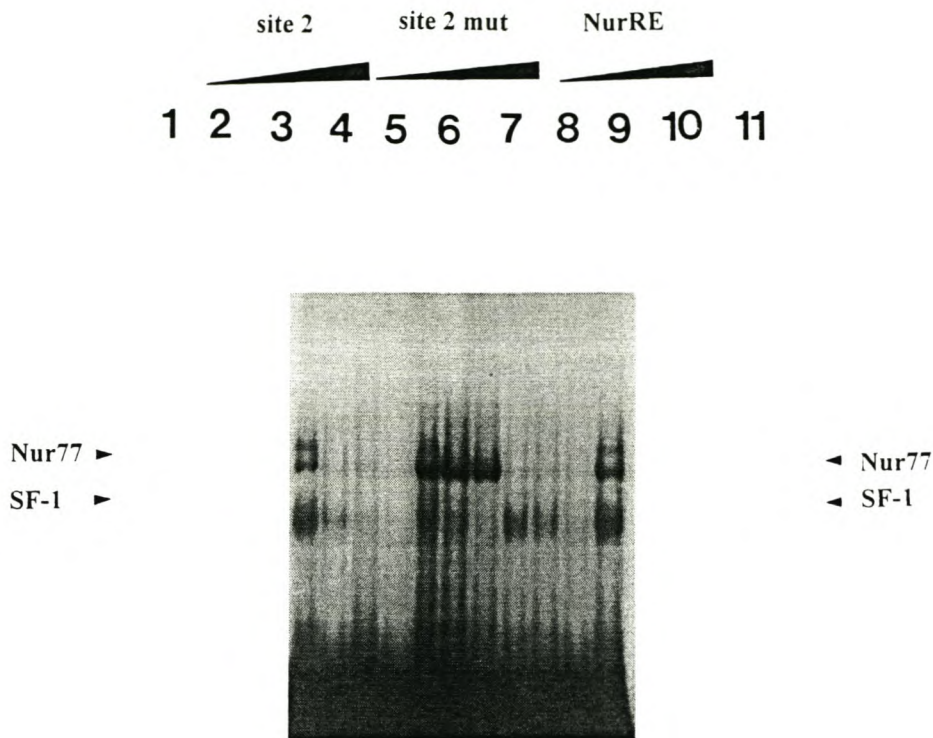


Figure 3.13: Autoradiograph of a gel mobility shift assay performed with α T3-1 nuclear extracts (batch 2) and the site 2 probe, in the absence and presence of varying concentrations of unlabelled competitor oligonucleotides. All the competitors were used at 5, 25 or 125 fold molar excess. In lanes 1 and 11, no competitors were added. Lanes 2 to 4 contain increasing amounts of site 2 competitor (site 2), lanes 5 to 7 contain mutated site 2 competitor (site 2 mut) and lanes 8 to 10 contain NurRE competitor (NurRE). The arrows denote the SF-1 and Nur77 protein complexes.

3.5: Regulation of SF-1 mRNA levels in the α T3-1 cell line by forskolin

A myriad of instances where SF-1 and cAMP are both involved in gene regulation were discussed in the introduction. A consensus CRE is usually absent from the promoters of these genes, and the cAMP effect often localizes directly to a SF-1 binding site in the promoters of target genes. These facts prompted an investigation into the possible effect of cAMP on SF-1 levels in α T3-1 cells.

α T3-1 cells were cultured under conditions similar to those under which cells were cultured for the first nuclear extracts preparation (i.e. the number of passages that the cells have undergone could have exceeded 10). The cells were incubated with increasing concentrations of forskolin for 18 hours and total RNA was isolated. The incubations were done in duplicate, i.e. the duplicate samples that were electrophoresed on the agarose gel before blotting were from independent incubations. The RNA was subjected to Northern blotting, after which the membrane was probed with the full-length human SF-1 cDNA. Figure 3.14, panel A, shows the autoradiograph of the membrane probed for SF-1 mRNA, and panel B shows the autoradiograph of the same membrane probed for β -actin mRNA levels, in order to correct for inconsistent loading of RNA onto the agarose gel. The autoradiographs were subjected to densitometric scanning and the values obtained for the SF-1 bands were normalized by dividing them by their corresponding β -actin values. The results of the statistical analysis of the normalized values can be seen in figure 3.14, panel C.

From panel A it appears that SF-1 mRNA in α T3-1 cells increased in a concentration-dependent manner in response to stimulation by forskolin. In panel B, the β -actin mRNA bands from the different incubations do not seem to differ in intensity. Therefore, from these results it is already clear that the concentration-dependent effect is real and not caused by inconsistent loading. The bar graph in panel C confirms this. When the respective normalized values obtained for the 10 μ M and 100 μ M forskolin incubations were compared to the control value, the increases were both

statistically significant ($P<0,001$, see ** in figure 3.14(C)). When the value for 10 μM forskolin was compared with that of 100 μM forskolin, the latter was statistically significantly greater ($P<0,005$) than the former.

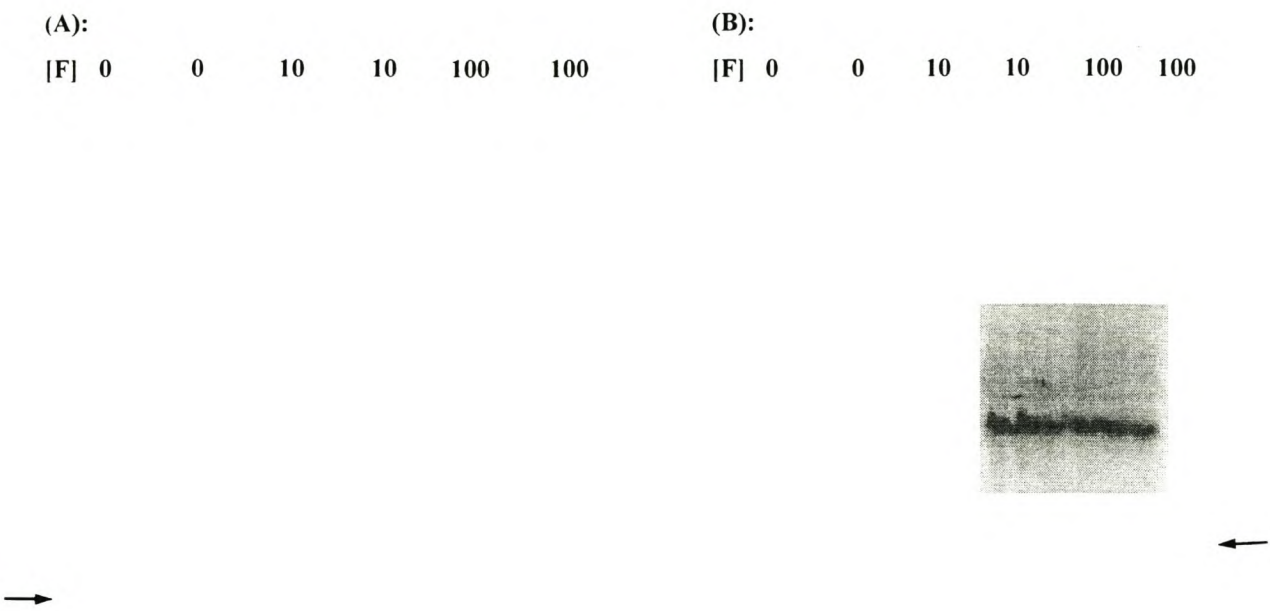


Figure 3.14 (A): Autoradiograph of a Northern blot analysis performed on total RNA isolated from $\alpha\text{T3-1}$ cells. The full-length human SF-1 cDNA was used as radiolabeled probe. The concentration of forskolin ([F]) in micromolar with which the cells were incubated before RNA isolation is indicated above the figure. The cells were incubated with forskolin for 18 hours. **(B):** To correct for loading differences in each well, the membrane was stripped and reprobed for β -actin. The arrows in panels A and B denote the SF-1 mRNA (2,9 kb) and β -actin mRNA bands, respectively.

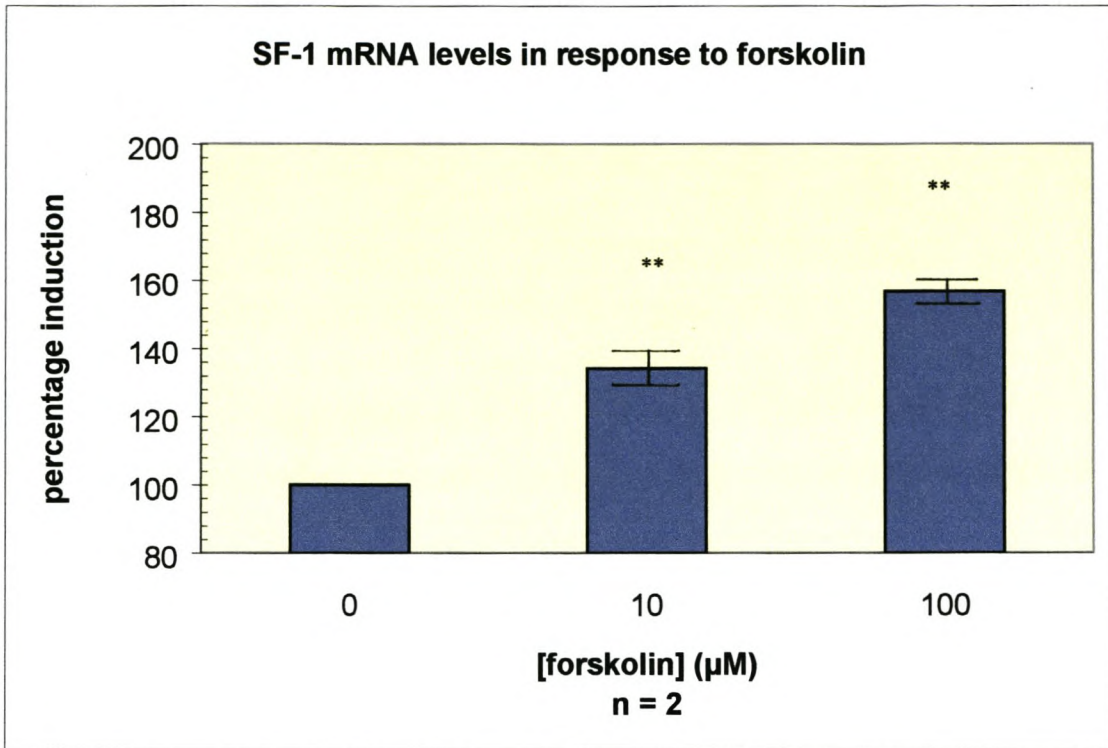


Figure 14 (C): Statistical analysis of the results of the Northern blot presented in panels A and B. The bands on the two autoradiographs were individually quantified by densitometric scanning and the values obtained for the SF-1 mRNA bands were normalized by dividing by the corresponding β -actin value. The normalized values were analysed using one-way ANOVA, and the control incubation (α T3-1 cells in the absence of forskolin) was taken as 100%. ** = $P < 0,001$.

Chapter 4: Discussion and Conclusions

4.1) The binding of SF-1 and Nur77 proteins to sites 1 and 2

A central question in this thesis was to identify the α T3-1 nuclear proteins binding to site 1 and site 2 in the mouse GnRH receptor promoter. The results of the gel mobility shift assays presented in figures 3.1 and 3.2 showed that several α T3-1 nuclear proteins from the first batch of extracts formed complexes with both the site 1 and site 2 probes. However, the anti-SF-1 antibody failed to detect the presence of SF-1 protein in any of these complexes. The fact that *in vitro* transcribed-translated SF-1 formed a complex with both probes suggested that SF-1 protein could bind to these sites, but this did not necessarily hold true for endogenous SF-1 protein. Furthermore, no SF-1-DNA complexes were identified by the anti-SF-1 antibody when the first batch of nuclear extracts was used in a gel mobility shift assay with a confirmed SF-1 binding site as probe (figure 3.3). These results strongly suggested that this batch of nuclear extracts did not contain functional SF-1 protein capable of binding to its specific binding site, either because SF-1 protein was not expressed in the cell line, or because the functional protein was damaged or lost during the extraction process. Alternatively, the possibility existed that SF-1 protein was actually present in one of the complexes, but that it formed part of a multi-protein-DNA complex. Such a complex might shield the DNA-binding domain of SF-1 and prevent the antibody from recognizing SF-1 protein in the complex. This possibility was tested by investigating whether or not the anti-SF-1 antibody was able to recognize *in vitro* transcribed-translated SF-1 protein in the presence of α T3-1 nuclear proteins. From figure 3.4 it is clear that the antibody was indeed able to do so. Taken together, these results showed that SF-1 protein is not present in the first batch of α T3-1 nuclear extracts. In contrast, figures 3.7 and 3.8 clearly show that SF-1 protein is present in the second batch of α T3-1 nuclear extracts, and that it can recognize and bind to both sites 1 and 2. SF-1 protein in α T3-1 nuclear extracts has been previously shown by others to bind to site 2 (Duval, Nelson, Clay, 1997), but this is the first report of SF-1 protein binding to site 1.

Unexpectedly, Nur77 protein was shown to be expressed in α T3-1 cells, and to bind to both sites 1 and 2. SF-1 and Nur77 proteins appear to bind to these sites in a mutually exclusive manner, since none of the DNA-protein complexes formed in the gel mobility shift assays were found to contain both proteins. This is indicated by the fact that none of these complexes were recognized by both the anti-SF-1 antibody and the anti-Nur77 antiserum. The mutually exclusive binding of SF-1 and Nur77 proteins to sites 1 and 2 is consistent with the fact that SF-1 protein appears to bind to DNA strictly as a monomer (Wilson *et al.*, 1993). Sites 1 and 2 are nuclear receptor half-sites, and can therefore only accommodate monomeric binding. Furthermore, no reports of protein-protein interactions between SF-1 and Nur77 have ever been published, and the two proteins are therefore unlikely to form heterodimers in this case.

The results of the competition assays (figures 3.10 to 3.13) show that SF-1 and Nur77 proteins have different DNA binding specificities. This is shown by the finding that mutations introduced into sites 1 and 2 abolished SF-1 protein binding to both oligonucleotides, but not Nur77 protein binding. Furthermore, it is interesting to note that the NurRE competitor competed equally for SF-1 protein and Nur77 protein bound to both the site 1 and site 2 probes (figure 3.11, lanes 8 to 10, and figure 3.13, lanes 8 to 10). This is curious, since one would expect a Nur77 binding site to compete for Nur77 protein binding more strongly than it would for SF-1 protein binding. This competitor represents a dimeric Nur77 binding site, and it should be investigated whether or not similar results would be obtained if a monomeric Nur77 binding site (NBRE) (Wilson *et al.*, 1991) is used as competitor.

From the competition assay results presented in figures 3.10 to 3.13, some general conclusions can be drawn about the relative affinity of SF-1 protein and Nur77 protein for the different oligonucleotides. Even though the four experiments of which the results are presented in figures 3.10 to 3.13 were not performed in parallel, they were performed with the same batch of α T3-1

nuclear proteins and with radiolabelled oligonucleotide probes with similar specific activities. Therefore, autoradiographs where the respective bands have similar intensities (i.e. figures 3.11 to 3.13) can be compared. These results indicate that SF-1 protein binds to sites 1 and 2 with similar affinity. It also appears as if Nur77 protein has approximately the same affinity for sites 1 and 2. However, since no information is available about the relative concentrations of SF-1 and Nur77 proteins in α T3-1 nuclear extracts, and the DNA binding affinities of the two proteins for a specific probe, one cannot determine from these results the relative affinities of Nur77 and SF-1 for site 1 or for site 2. However, the results do show that, for the second batch of α T3-1 nuclear extracts, both sites 1 and 2 are preferentially bound by SF-1 protein, rather than Nur77 protein.

Previous results obtained in this laboratory indicated that mutation of either site 1 or site 2 alone did not have any significant effect on transcription from the mouse GnRH receptor promoter in α T3-1 cells, but mutation of both sites simultaneously led to a significant decrease in promoter function (Styger, 2001). Originally, this was interpreted as an indication of functional redundancy between the two sites. The "loss" of one site could still allow SF-1 protein to perform its function and activate the GnRH receptor promoter, but when neither of the two sites is available for SF-1 protein binding, transcriptional activation is impaired. However, the finding that Nur77 protein is expressed in α T3-1 cells and is able to bind to the mutated SF-1 binding sites, as shown by the competition assays in figures 3.10 to 3.13, urges one to reconsider this interpretation. Bearing in mind that Nur77 protein binding to the mutated sites could have a transcriptional effect, it is probably necessary to re-investigate the GnRH receptor promoter function in α T3-1 cells.

A strange aspect of the results shown in figures 3.7 and 3.8 is the finding that the presence of the anti-Nur77 antiserum dramatically and reproducibly increased the intensity of the SF-1 complex. One might argue that, if SF-1 and Nur77 protein compete for the same binding site on the probe, more SF-1 protein would bind to the probe in the presence of anti-Nur77 antiserum than in the

absence thereof, due to the "removal" of competing Nur77 protein by the antibody. However, this is unlikely, since the anti-Nur77 antiserum does not "remove" Nur77 protein. The anti-Nur77 antiserum does not prevent Nur77 protein binding to the probe, as is the case with the anti-SF-1 antibody, but supershifts the DNA-protein complex, thereby still allowing Nur77 protein to bind to the probe. It is therefore unlikely that this effect is due to an increase in the concentration of free probe in the presence of anti-Nur77 antiserum. Another possibility is that one or more of the serum proteins present in the anti-Nur77 antiserum could act to stabilize the SF-1-DNA complex. Both the anti-SF-1 and anti-COUP-TF antibodies are purified IgG, whereas the anti-Nur77 antibody is contained in rabbit serum, along with a full complement of serum proteins. This could explain why the effect is only seen with anti-Nur77 antiserum, and not with the other antibodies. The effect was also seen with *in vitro* transcribed-translated SF-1, in the presence of anti-Nur77 antiserum (Schipper *et al.*), and is therefore not limited to SF-1 protein from α T3-1 cells.

4.2) The expression of SF-1 and Nur77 protein in the α T3-1 cell line

The presence of SF-1 protein in the first batch of α T3-1 nuclear extracts was investigated by Western blot analysis. The first Western blot analysis was performed with the same antibody as was used in the gel mobility shift assays, namely the antibody against the SF-1 DNA binding domain, purchased from Upstate Biotechnology. In figure 3.5, no signal corresponding to 53 kDa can be observed in the lanes containing α T3-1 nuclear proteins, consistent with the result that SF-1 protein is absent in the first batch of α T3-1 nuclear extracts. However, a signal corresponding to 66 kDa can be observed in these lanes. It is possible that this signal is caused by a member of the nuclear receptor superfamily that has a DNA-binding domain similar to that of SF-1, which therefore cross-reacts with the anti-SF-1 antibody.

However, it is clear from figure 3.5 that this antibody is not specific enough to yield a conclusive Western blot result. Apart from the signal obtained with the unidentified 66 kDa nuclear protein, a

clear signal corresponding to 53 kDa is also present in the negative control lane. This signal possibly represents a reticulocyte lysate protein with a DNA binding domain similar to that of SF-1. This protein could, as already explained in the case of the unidentified 66 kDa α T3-1 nuclear protein, cross-react with the anti-SF-1 antibody and produce a false signal. The fact that a signal of the same size and intensity is present in the positive and negative control lanes indicates that the protein producing the signal in the positive control lane is not SF-1. The antibody against the SF-1 DNA binding domain also failed to recognize SF-1 protein in the second batch of α T3-1 nuclear extracts in a Western blot experiment (not shown). These findings show that this antibody is unable to recognize SF-1 protein under the conditions of the Western blot experiment.

When the Western blot was repeated with an antibody against the whole protein, the results looked markedly different (figure 3.6). With this antibody, only a faint signal was observed in the negative control lane. Additionally, no signal corresponding to 66 kDa was observed in the lanes containing α T3-1 nuclear proteins from either batch 1 or batch 2 extracts. More importantly, though, a strong signal corresponding to 53 kDa was observed in the lane containing α T3-1 nuclear proteins from the second batch of nuclear extracts, whereas no signal was observed in the lane containing α T3-1 nuclear proteins from the first batch. This experiment therefore clearly shows that SF-1 protein is undetectable in the first batch of α T3-1 nuclear extracts, but present in the second batch. Although Ingraham *et al.* previously showed that SF-1 protein is expressed in α T3-1 cells (Ingraham *et al.*, 1994), the results presented in this thesis clearly show that expression levels of SF-1 can vary significantly for different batches of α T3-1 cells.

The striking differences in the Western blot results obtained with the two different anti-SF-1 antibodies can probably be explained by their different specificities. The antibody from Upstate Biotechnology was raised against the DNA binding domain of SF-1, whereas the antibody from Ken Morohashi was raised against the whole protein. It is quite possible that the denaturing

conditions of the SDS gel electrophoresis could change the three-dimensional appearance of the protein to such an extent that the Upstate Biotechnology antibody can no longer recognize the DNA-binding domain. On the other hand, the anti-SF-1 antiserum from Ken Morohashi is a mixture of antibodies against many different epitopes on the surface of the protein. Therefore, even under denaturing conditions, the antibody is likely to retain the ability to recognize at least a few of these epitopes, resulting in a more specific signal. Naturally, these concerns do not apply to the non-denaturing conditions of gel mobility shift assays, explaining why the Upstate Biotechnology antibody is specific in these assays, even though this is not the case in Western blot experiments.

The first batch of nuclear extracts, in which SF-1 protein cannot be detected, contains two Nur77 protein isoforms, whereas the second batch contains only one isoform (see figure 3.9). These two Nur77 protein products may be the result of alternative splicing of primary transcripts, the use of different transcriptional or translational start sites, or altered post-translational modification. These factors can all be influenced by changes in cellular and metabolic status. As discussed before, the α T3-1 cells from which the first batch of nuclear extracts was prepared, had been maintained through an unknown number of passages, over a long period of time. The cells that were cultured to prepare the second batch of extracts from were grown from a fresh stock, and they only went through a few passages. From the Western blot results shown in figures 3.6 and 3.9, it is clear that the expression levels of SF-1 protein and the larger Nur77 protein isoform are regulated by these differences in cell culture conditions. Furthermore, it is interesting to note that the larger Nur77 protein isoform is only expressed in the absence of SF-1 protein. Therefore, the same culture conditions that inhibit SF-1 protein expression induce the expression of this Nur77 protein isoform. Alternatively, it is possible that SF-1 protein might inhibit the expression of the larger Nur77 protein isoform. The smaller Nur77 protein isoform appears to be constitutively expressed and is not influenced by the different culture conditions.

The detection of Nur77 protein in α T3-1 nuclear extracts was quite unexpected, since this nuclear receptor has not previously been reported to be expressed in a pituitary gonadotrope cell line. The presence of Nur77 protein was confirmed by several lines of evidence presented in this study. The anti-Nur77 antibody was shown to be specific for Nur77 protein, as judged by its lack of cross-reactivity with SF-1 protein in gel mobility shift assays and Western blot experiments. This antibody also did not cross-react with any of the other α T3-1 nuclear protein complexes in the gel mobility shift assays, nor did it recognize any other α T3-1 nuclear proteins in the Western blot experiment. Furthermore, the antibody was able to detect Nur77 protein in the positive control CCRF-CEM nuclear extracts, in both the gel mobility shift assays and the Western blot. In the Western blot, the Nur77 protein bands in the lanes containing α T3-1 nuclear extracts were clear, sharp bands, with no other bands visible in these lanes. Neither the Nur77 protein in the positive control nuclear extracts, nor the Nur77 protein in α T3-1 nuclear extracts, cross-reacted with the anti-SF-1 or anti-COUP-TF antibodies, clearly showing that this protein is not SF-1 or COUP-TF. In addition, the Nur77 protein complex formed by α T3-1 nuclear extracts and the Nur77 protein complex detected with the CCRF-CEM positive control nuclear extracts both had the same mobility in the gel mobility shift assays. Furthermore, the protein in the α T3-1 nuclear extracts detected by the anti-Nur77 antiserum had the same mobility on the SDS gel as Nur77 protein in the positive control nuclear extracts.

The α T3-1 cells used in these experiments were shown by others in our laboratory to express GnRH receptor (Styger, 2001) by a Northern blot analysis on the same total RNA isolated from these cells as was used in the Northern blot presented in figure 3.14. Since GnRH receptor expression is regarded as a pituitary gonadotrope-specific marker, this result shows that the cells are indeed gonadotrope cells. One could argue that the α T3-1 cells that were used to prepare the nuclear extracts were contaminated with a pituitary corticotrope cell line, which expresses Nur77 protein (Okabe *et al.*, 1998). However, such contamination could not have occurred in our laboratory, since

no corticotrope cell lines are maintained in our tissue culture facility. Corticotropes are the only pituitary cells that express the POMC gene (Okabe *et al.*, 1998). Results from a Northern blot analysis on the same batch of total RNA used in the two Northern blot experiments mentioned above indicated that there is no POMC mRNA present in these cells (result not shown) (Schipper, 2001). This result shows that the gonadotrope cell culture is not contaminated with corticotropes that might produce a false positive signal for Nur77 protein on the Western blot. When all of these facts are taken into consideration, they strongly suggest that Nur77 protein is indeed expressed in the α T3-1 cell line.

It is interesting to speculate on the physiological significance of Nur77 expression in a gonadotrope cell line. It had been well established by others that Nur77 protein is expressed in corticotropes, where it combats the negative feedback of increased glucocorticoid levels on the HPA axis under chronic stress (Okabe *et al.*, 1998). If one assumes, from the finding that Nur77 protein is expressed in a gonadotrope cell line, that it is also expressed in primary gonadotrope cells, this strongly suggests that Nur77 may also be involved in the regulation of the HPG axis. It is well known that reproductive function is (and needs to be) compromised under stress conditions, in order to protect both the parent and the offspring. The mechanism by which this control is mediated is not clear. The presence of Nur77 in gonadotropes could have more than one possible implication. One possibility could be that, under stress conditions, Nur77 contributes to the down-regulation of GnRH receptor expression, resulting in decreased reproductive potential. Alternatively, Nur77 could serve as a positive regulator of GnRH receptor expression, to maintain basal levels of essential functions of the reproductive system, such as gonadal steroidogenesis, under stress conditions. This may be analogous to the situation in the adrenals, where Nur77 has a positive effect on adrenal steroidogenesis (Crawford *et al.*, 1995). Nur77 could therefore very well be involved in cross-talk mechanisms between the HPA and HPG axes.

4.3) Transcriptional regulation of the GnRH receptor gene by various signal transduction pathways

In our laboratory, others have shown that the GnRH receptor promoter responds to stimulation of the PKA pathway by forskolin in α T3-1 cells, by a mechanism that involves site 1. It was shown by Northern blotting that endogenous GnRH receptor levels in α T3-1 cells increase in response to forskolin (Styger, 2001). A question that arose from these experiments was whether this effect was a direct or an indirect effect. It was therefore necessary to determine the effect of the PKA pathway on the expression levels of SF-1. Figure 3.14 clearly shows that SF-1 mRNA levels in α T3-1 cells increase in a dose-dependent fashion in response to stimulation of the PKA pathway by forskolin. This appears to be similar to the situation in the ovary, where SF-1 expression levels increase in response to cAMP (Michael *et al.*, 1995; Zeitoun *et al.*, 1999). In contrast, a different mechanism of SF-1 regulation in response to PKA stimulation appears to function in the adrenals, where SF-1 protein activity is upregulated by cAMP stimulation, while expression levels are not influenced (Zhang and Mellon, 1996). Since SF-1 protein has been shown in this thesis to bind to both site 1 and site 2 in the GnRH receptor promoter, it is very likely that stimulation of the PKA pathway will have a positive effect on GnRH receptor promoter activity in α T3-1 cells, via increased SF-1 binding to these two sites. Thus, taken together, these results indicate that GnRH receptor transcription is upregulated in response to stimulation of the PKA pathway in α T3-1 cells, via an indirect mechanism involving upregulation of SF-1 expression.

Even though the PKA pathway was shown to have a positive effect on GnRH receptor promoter activity in α T3-1 cells, it is not known what signals trigger the activation of the pathway in this cell line. It has been established by several authors that the response of the mouse GnRH receptor promoter to GnRH in α T3-1 cells is mediated via the PKC pathway, and not the PKA pathway (White *et al.*, 1999; Norwitz *et al.*, 1999). However, Lin and Conn have shown the PKA pathway to be involved in the GnRH response in GGH₃ cells (Lin and Conn, 1998). Furthermore, the response

of the glycoprotein hormone α -subunit promoter to GnRH stimulation in α T3-1 cells appears to be mediated via a CRE (Burrin *et al.*, 1998). Thus, although the PKA pathway does not appear to be involved in the response of the GnRH receptor promoter to GnRH in α T3-1 cells, the PKA pathway may still be involved in mediating the effects of some other signals in these cells. It is also important to note that SF-1 expression levels in α T3-1 cells were found by others not to increase in response to GnRH stimulation (Tremblay and Drouin, 1999), and it is therefore unlikely that GnRH is involved in mediating the effects of PKA stimulation via changes in SF-1 levels.

Many genes, such as the steroidogenic enzyme genes, are responsive to SF-1 and to stimulation of the PKA pathway (Morohashi *et al.*, 1992). In most of these genes, no CRE can be identified in the promoter, and the SF-1 binding site is responsible for mediating the full response of the promoter to the PKA pathway. In contrast, the mouse GnRH receptor promoter does contain a CRE, albeit an atypical one (Maya-Núñez and Conn, 1999). This is surprising, since site 1 has been shown to mediate the full response of the GnRH receptor promoter to the PKA pathway in α T3-1 cells (Styger, 2001). The atypical CRE has been shown to be involved in the response of the GnRH receptor promoter to the PKA pathway in GGH₃ cells (Maya-Núñez and Conn, 1999), and it would thus be interesting to investigate its role in α T3-1 cells. The possibility exists that the CRE is non-functional in α T3-1 cells, or that it makes only a minor contribution to the PKA response of the GnRH receptor in this cell line.

It has been shown in this thesis that SF-1 and Nur77 proteins compete for binding to both site 1 and site 2 in the GnRH receptor promoter. These two proteins also compete for binding to the same site in the cytochrome P450 21-hydroxylase gene, which is expressed in the adrenal cortex. In these cells, Nur77 expression is increased in response to cAMP stimulation, while SF-1 protein appears to be constitutively expressed (Crawford *et al.*, 1995). As found in this thesis, this is in contrast to the situation in α T3-1 cells, where SF-1 expression increases in response to stimulation by forskolin,

and is also influenced by culture conditions. It has also been shown that Nur77 expression in α T3-1 cells is regulated by culture conditions. Additionally, preliminary results obtained in our laboratory indicate that Nur77 mRNA levels in α T3-1 cells increase in response to forskolin (Sadie *et al.*, manuscript in preparation). The significance of SF-1 and Nur77 proteins competing for two sites in the GnRH receptor promoter is not clear. Hence, more experiments need to be done to elucidate the mechanism whereby these two proteins affect GnRH receptor promoter activity, and their possible roles in mediating the response of the promoter to the PKA pathway.

4.4) The possible role of COUP-TF in GnRH receptor gene regulation

Several instances were discussed in the introduction where SF-1 and COUP-TF proteins compete for binding to the same nuclear receptor binding site. SF-1 and COUP-TF proteins bind in a mutually exclusive manner to sites in the promoters of the bovine cytochrome P450 17 α -hydroxylase gene (Bakke and Lund, 1995), the DAX-1 gene (Yu *et al.*, 1998), and the aromatase gene (Zeitoun *et al.*, 1999), where COUP-TF inhibits the activation of these genes by SF-1. Given the fact that COUP-TF appears to be expressed in α T3-1 cells (Yu *et al.*, 1998), it was necessary to investigate the binding of COUP-TF to sites 1 and 2. The specific anti-COUP-TF antibody did not detect the presence of COUP-TF protein in any of the DNA-protein complexes formed between the site 1 and site 2 probes and α T3-1 nuclear extracts from the second batch (figure 3.7 and 3.8). However, the presence of COUP-TF protein was not determined by Western blotting for either the first or the second batch of nuclear extracts. The finding that SF-1 and Nur77 protein expression varied significantly from one batch of α T3-1 cells to the next indicates that it cannot be assumed that COUP-TF protein is expressed in α T3-1 cells under our culture conditions.

It is important to note that the anti-COUP-TF antiserum was only used against α T3-1 nuclear proteins in gel mobility shift assays. No positive control, either in the form of *in vitro* transcribed-translated COUP-TF or another mixture of proteins previously confirmed to contain COUP-TF, was

included in these assays. Hence, the possibility exists, although unlikely, that the anti-COUP-TF antiserum is non-functional under the conditions of the gel mobility shift assays, and that one of the DNA-nuclear protein complexes does contain COUP-TF protein.

Assuming that the anti-COUP-TF antibody is functional, the absence of a COUP-TF-DNA complex in the gel mobility shift assays suggests either that COUP-TF protein is not present in the second batch of α T3-1 nuclear extracts, or that it is present, but not recognizing sites 1 and 2. It is also possible that COUP-TF protein will only bind to the two sites in the absence of SF-1 protein. It would have been interesting to investigate this possibility with the first batch of nuclear extracts, but unfortunately this batch was used up by the time this issue became apparent. Furthermore, the possibility exists that COUP-TF in α T3-1 cells preferentially binds to DNA as a dimer, as is the case in many other instances (reviewed in Sugiyama *et al.*, 2000). In this respect, it might be very significant that the GnRH receptor promoter contains two nuclear receptor binding sites. It is possible that, through DNA looping, COUP-TF homodimers bind to sites 1 and 2 simultaneously. COUP-TF homodimers would therefore be unable to bind to the individual binding sites in the gel mobility shift assays.

In summary, the results in figures 3.7 and 3.8 suggest that COUP-TF is not involved in the transcriptional regulation of the GnRH receptor promoter in α T3-1 cells. However, many outstanding questions, such as the expression of COUP-TF protein in our batch of α T3-1 cells, need to be addressed before this can be confirmed.

4.5) The effect of cell culture conditions on gene expression

When cells have undergone a number of passages, the selective pressure exerted by the cell culture conditions tends to select for more robust and vigorous cell types. Continuous proliferation (maintaining cells through numerous passages) may also select for undifferentiated precursor cells.

This could either be the result of undifferentiated cells overgrowing fully differentiated ones, or the result of the absence of appropriate inducers maintaining expression of specialized functions. The first possibility results in the irreversible loss of differentiated functions, and is referred to as dedifferentiation. The second scenario implies that the expression of specialized functions can be restored upon administration of the appropriate inducer, and is referred to as deadaptation (Freshney, 1982).

The cell culture environment, especially involving cells of the nervous and endocrine systems, also lacks the hormonal and nutritional components needed for maintaining cellular homeostasis and communication. Cell-cell and cell-matrix interactions are further reduced because of the two-dimensional structure of the culture. The metabolism of cultured cells might therefore not always be representative of the tissue from which the cells were originally derived. Hence, it is essential to provide the required hormones, growth factors and nutrients to maintain specialized functions in the cells. Failure to do so will result in altered gene expression, which will ultimately influence the results obtained from the cell culture system (Freshney, 1982).

These inconsistencies in cell culture systems often cause results obtained from tissue culture experiments to be viewed with skepticism. It is therefore very important to be aware of the limitations of the system. More importantly, it is necessary to take meticulous care in reproducing the culture conditions from one experiment to the next, to ensure consistency of results. The importance of this is highlighted by the fact that SF-1 protein is undetectable in one batch of α T3-1 cells but readily detectable in another, and that different Nur77 protein isoforms are expressed under different growth conditions. These differences in gene expression might have profound implications for transfection experiments and functional promoter studies performed in the α T3-1 cell line.

4.6) The role of nuclear receptors in transcriptional regulation

As already discussed earlier, SF-1 and Nur77 proteins compete for a common binding site in the cytochrome P450 21-hydroxylase promoter in adrenal cells. Additionally, SF-1 and COUP-TF protein compete for binding sites in the promoters of several genes. The SF-1 binding site in the bovine proximal aromatase promoter can also be recognized by several nuclear receptors (Michael *et al.*, 1995). Thus, there are many instances noted in the literature where SF-1 binding sites can be occupied by other nuclear receptors, resulting in modulation of transcription. This raises the question of which other nuclear receptors might be binding to sites 1 and 2 in the GnRH receptor promoter.

Table 1: Binding sites for SF-1 and other orphan nuclear receptors

SF-1 binding sites	
rat P450 aromatase gene (Lynch <i>et al.</i> , 1993)	5' CCA AGG TCA 3'
mouse P450 21-hydroxylase (Rice <i>et al.</i> , 1990)	5' CAA AGG TCA 3'
human P450 side chain cleavage gene (Monté <i>et al.</i> , 1998)	5' TCA AGG CCA 3'
glycoprotein hormone α -subunit: human	5' ACA AGG TCA 3'
mouse and rat (Horn <i>et al.</i> , 1992)	5' TCA AGG TCA 3'
mouse GnRH receptor: site 1	5' CCA AGG ACA 3'
site 2 (Albarracin <i>et al.</i> , 1994)	5' TGA AGG CCA 3'
COUP-TF binding sites	
chicken ovalbumin gene (Wang <i>et al.</i> , 1989)	5' CAA AGG TCA 3'
consensus direct repeat site (H. Lin <i>et al.</i> , 1999)	5' C AGG TCA C AGG TCA 3'
Nur77 binding sites	
consensus site (NBRE) (Wilson <i>et al.</i> , 1991)	5' AAA AGG TCA 3'
NurRE (Philips <i>et al.</i> , 1997)	5' TGA TTT TAC CTC CAA ATG CCA 3'

From table 1 it can be seen that the central bases 5' AGG 3' are absolutely conserved among all of the nuclear receptor binding sites, except for the NurRE. The two central cytosine residues complementary to the two guanine residues in the opposite strand have been determined to be absolutely essential for recognition by SF-1. Mutating these cytosines to thymidines completely abolishes SF-1 binding (Horn *et al.*, 1992). Apart from the central 5' AAGG 3' and the 5' CA 3' at the 3' end, promoter elements that are generally believed to be SF-1 binding sites seem to be able to tolerate a fairly large extent of degeneracy within the 9 base-pair binding site sequence.

To identify consensus binding sequences for COUP-TF and Nur77 is more complicated than for SF-1. Both COUP-TF and Nur77 can bind to DNA as monomers (Wilson *et al.*, 1993; Bakke and Lund, 1995), homodimers (Philips *et al.*, 1997; Sugiyama *et al.*, 2000) and RXR heterodimers (reviewed in Maruyama *et al.*, 1998 and Sugiyama *et al.*, 2000). They can even heterodimerize with each other (Wu *et al.*, 1997), and each one of these combinations recognizes DNA elements of unique structure and sequence. The COUP-TF binding site originally identified in the chicken ovalbumin promoter was a single nuclear receptor half-site (Wang *et al.*, 1989), but it was later established that COUP-TF actually preferentially binds as homodimers (reviewed in Sugiyama *et al.*, 2000) to direct repeats of the nuclear receptor core half-site (H. Lin *et al.*, 1999). Much the same is also true for Nur77. The original consensus site was a single extended half-site binding Nur77 monomers (Wilson *et al.*, 1993), but the Nur77 response element in the corticotrope-specific POMC gene consists of inverted repeats of the extended half-site and binds Nur77 homodimers (Okabe *et al.*, 1998).

Judging from the information in table 1, it is clear that even just a single base-pair substitution in a nuclear receptor binding site could result in the binding of a different nuclear receptor than what was initially believed to bind there. An “imperfect” SF-1 site could easily function as an “imperfect” Nur77 or COUP-TF site. Thus, a given site could bind several different receptors, albeit

with different affinities. This does not mean that sites that exclusively bind a specific receptor do not exist. However, with the information currently available, one should be wary of predicting that a nuclear receptor binding site is an exclusive binding site for SF-1 or Nur77, or any other orphan nuclear receptor.

The results presented in this thesis show that both SF-1 and Nur77 proteins bind *in vitro* to sites 1 and 2 in the mouse GnRH receptor promoter. This provides another example of promoter elements that can be occupied by more than one nuclear receptor. Originally, it was assumed that SF-1 is the only orphan nuclear receptor involved in the regulation of the GnRH receptor gene via these sites. Other authors did not appear to investigate the presence of other members of the nuclear receptor superfamily in the α T3-1 cell line, or the binding of these factors to sites 1 and 2. However, the many instances discussed in the introduction where two or more nuclear receptors can recognize the same binding site, together with the results presented here, strongly indicates the importance of broader investigation of nuclear receptors and their binding sites when investigating the transcriptional regulation of a specific gene in a given cellular context.

Quite often, papers are published where the investigators identify a *cis* element in the promoter of a gene through functional promoter studies. When this *cis* element is mutated or deleted from the promoter, a specific transcriptional effect is observed. By virtue of sequence analysis and comparison to “consensus sites”, candidate proteins are suggested that might recognize the element. A reporter construct containing the element under investigation is prepared, and co-transfected together with an expression construct of the candidate protein. When a positive result is obtained, this is regarded as conclusive proof that transcription of the gene is regulated by the candidate protein.

However, all the instances mentioned in the literature of nuclear receptors competing for the same binding site, and the results presented here, together with the sequence similarities shared by nuclear receptor binding sites, strongly warns against drawing these kinds of conclusions. In addition, the proteins binding to specific DNA sequences are often identified through *in vitro* DNA binding studies, and these DNA-protein interactions might not always be occurring *in vivo*.

Because nuclear receptor binding sites have such similar sequences, overexpression of any protein that could bind to a specific site would almost invariably lead to its binding, purely on the basis of competition. This does not mean that it would bind there under physiological conditions in the primary tissue. Furthermore, overexpressing one factor in a cell line would almost invariably titrate other transcription factors and components of the transcriptional machinery. This could completely disrupt the balance of factors and influence the functioning of other factors in the nucleus. Transcriptional regulation by nuclear receptors is dependent on relative receptor concentrations, relative binding affinities, competition for binding sites, promoter contexts and chromatin structure. The actual levels of expression of a target gene in a specific tissue is the result of a delicate interplay between different receptors, as well as the conditions determining their biological activity for binding to specific promoter elements.

The results presented in this thesis should be viewed in the context of these considerations. While strong evidence is provided that SF-1 and Nur77 proteins are involved in the transcriptional regulation of the mouse GnRH receptor gene via sites 1 and 2, it cannot be assumed that this situation exists *in vivo* in α T3-1 cells, or indeed in primary pituitary tissue.

4.7) Future perspectives

When regarding the results presented in this thesis in the light of results published by others, some very interesting issues are raised. SF-1 and Nur77 proteins compete for binding to sites 1 and 2 in gel mobility shift assays, but that does not give any indication of which protein is occupying which site *in vivo* under various conditions. It is also not known what the functional significance of the binding of these two nuclear receptors to sites 1 and 2 is on GnRH receptor transcription. Before these questions can be answered, additional experiments need to be done, i.e. the relative concentrations of SF-1 and Nur77 protein in α T3-1 cells, and their relative affinities for sites 1 and 2, need to be determined. More specifically, their individual base pair requirements for binding to sites 1 and 2 need to be clarified. Once this is established, a new set of functional promoter studies should be performed in an attempt to delineate specific functions of SF-1 and Nur77 protein at the two sites. Furthermore, the functional roles of SF-1 and Nur77 in mediating the PKA response need to be delineated, and this can be done by performing these experiments in the absence and presence of forskolin. Additionally, the functional consequences of manipulation of the relative concentrations of SF-1 and Nur77 through overexpression of the receptors on promoter activity should be determined. It also needs to be determined whether the effect of the PKA pathway on the GnRH receptor promoter in α T3-1 cells is direct or indirect, i.e. whether it involves *de novo* protein synthesis or not. This can be determined by investigating the levels of endogenous GnRH receptor mRNA in response to PKA, in the absence and presence of cycloheximide, a protein synthesis inhibitor.

Earlier studies by Pheiffer and Styger suggest that SF-1 has a positive effect on GnRH receptor promoter activity, but it is not clear what possible effect Nur77 might have on promoter activity. Since Nur77 null mice have no impairment of reproductive function (Crawford *et al.*, 1995), it is possible that Nur77 performs a compensatory function under conditions where SF-1 expression is reduced. In this respect, it is important to be reminded of the finding that a second Nur77 protein

isoform is expressed in α T3-1 cells, under conditions where SF-1 protein is undetectable. It would be interesting to determine which of these Nur77 isoforms recognizes sites 1 and 2, and how the expression levels and DNA binding activities of the isoforms are influenced by the presence of SF-1 and the activation of the PKA pathway.

Results presented in this thesis, together with work done by others in our laboratory, show that GnRH receptor, SF-1 and Nur77 mRNA levels in α T3-1 cells all increase in response to forskolin (Styger, 2001; Sadie *et al.*, 2001). At this point, the extracellular signals that would be propagated by cAMP as second messenger are not known. From information in the literature, it appears unlikely that this signal is GnRH. It is necessary to identify the conditions under which the PKA pathway would be activated, and the physiological relevance of PKA pathway activation. It would also be necessary to further investigate the role of site 2, the CRE and other promoter elements in the PKA response in α T3-1 cells.

It is very important that the expression of various transcription factors in the α T3-1 cell line be investigated, through Northern and Western blotting. These experiments should focus on factors such as COUP-TF, DAX-1, and Ptx1. All three of these proteins are known to be expressed in the pituitary, but their expression in the α T3-1 cell line has not been investigated. As discussed in the introduction, both COUP-TF (Bakke and Lund, 1995; Yu *et al.*, 1998; Zeitoun *et al.*, 1999) and DAX-1 (Nachtigal *et al.*, 1998; Crawford *et al.*, 1998) have an inhibitory effect on SF-1 function in other systems. Ptx1 interacts with SF-1 to activate transcription of the LH β gene in gonadotropes, and is believed to be involved in facilitating the ligand-independent activity of SF-1 protein (Tremblay *et al.*, 1999). It would therefore be interesting to determine whether or not these factors are present in α T3-1 cells, since all of them could potentially influence SF-1 activity. Furthermore, it should be investigated whether any of these factors are present in the protein-DNA complexes formed in the gel mobility shift assays between the site 1 and site 2 probes and α T3-1 nuclear

extracts. It would also be interesting to investigate the relative expression levels of these factors in the absence and presence of forskolin, to shed light on their possible role in the PKA response. Additionally, the protein-protein interactions between these receptors and other co-factors, and their binding to sites 1 and 2, need to be elucidated.

Apart from the gonadotrope-specific markers, such as GnRH receptor and the glycoprotein hormone subunits, the profile of gene expression in the α T3-1 cell line is poorly characterized. It is essential to obtain more detailed information about this system, i.e. the expression of various nuclear receptors and other transcription factors, in order to understand the complex interplay between these different factors in regulating GnRH receptor gene expression and mediating the response to the PKA pathway.

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